

***Production and Characterization of PQQ- Dependent
Alcohol and Aldehyde Dehydrogenases on Assembly
of BioFuel Cells***

DISSERTATION FOR MASTER DEGREE IN BIOENGINEERING
SPECIALIZATION IN BIOLOGICAL ENGINEERING

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For my parents, especially to my mother...

*“Just a memory
Every dream is of you and me
If I wish upon a star
Well I hope that's where you are
When Heavens turn
You know you'll shine you're in my heart for all time
When Heaven turns you know you'll shine in worlds apart
Cause yeah you are my Star”*

Star, **Reamonn**

This dissertation is dedicated to my parents, for their love and endless support, especially to my mother, wherever she is.

September 2016

Declaração / Declaration

Declara, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Declare, under oath, that this work is original and that all non-original contributions were properly referenced with the source identification.

Porto, 16 setembro de 2016 | Oporto, 16th september 2016

(Ivo Dinis Leite Barros)

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ABTS	2,2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulphonic) Acid
AOx	Aldehyde Oxidase
BFCs	Biofuel Cells
BM	Basal Medium
BMIMDCA	1-Butyl-3-Methylimidazolium Dicyanamide (ionic liquid)
BOD	Biochemical Oxygen Demand cathode
CDH	Cellobiose Dehydrogenase
CF	Carbon Felt
Cox	Cyclooxygenase
DCPIP	2,6 – Dichlorophenol Indophenol
dcw	Dry cell weight
DET	Direct Electron Transference
EFCs	Enzymatic Fuel Cells
FAD+ / FADH	Flavin Adenine Dinucleotide (Oxidized / Reduced forms)
FDH	10-Formyltetrahydrofolate Dehydrogenase
GDH	Glutamate Dehydrogenase
GLY	Glycerol
GOX	Glucose Oxidase
GYC	Medium of Glucose, Yeast Extract and Calcium Carbonate
HRP	Horseradish Peroxidase
IL	Ionic Liquid

MAN	D-Mannitol
MET	Mediated Electron Transference
MFCs	Microbial Fuel Cells
MP-11	Microperoxidase-11
MP-8	Microperoxidase-8
NAD + /NADH	Nicotinamide Adenine Dinucleotide (Oxidized / Reduced forms)
OD	Optic Density
OOx	Oxalate Oxidase
OS Polymer	Osmium Redox Polymer
PDH	Pyruvate Dehydrogenase
PHFCs	Photochemical Fuel Cells
PMS	Phenazine Methosulfate
PQQ - ADH	Pyrroquinoline quinone - Dependent Alcohol Dehydrogenase
PQQ - ALDH	Pyrroquinoline quinone - Dependent Aldehyde Dehydrogenase
Ru(bpy)₃	Tris (Bipyridine) Ruthenium(II) Chloride
SCE	Saturated Calomel Electrode
TFE	Tetrafluoroethylene (TFE)
TIF	Tetrathiafulvalene
VK3	2-Methyl-L,4-Naphthoquinone (Vitamin K3)
YPM Medium	Medium of Yeast Extract, Peptone and D-Mannitol

NOMENCLATURE AND SYMBOLS

Symbol	Variable	Units
ΔV	Potential difference (voltage)	Volt (V)
μ	Specific growth rate	h^{-1}
A	Area	cm^2
Abs	Absorbance	(-)
C	Coulombs	-
Cp	Coulombic Efficiency	C (Eléctron/s)
F	Faraday's constant	96.485 C/mol
I	Intensity of current	Ampere (A)
K_s	Half-saturation constant for substrate	mg/L
L	Path light	cm
M	Concentration	mol /L (Molar)
MW	Molecular mass of the compound	g/mol
P	Power	Watt (W)
P_{DA}	Power Density by Surface Area	W/cm ²
P_{DV}	Power Density by Volume	W/m ³
R	Resistance	Ohms (Ω)
r	Ratio	cm
S	Substrate Concentration	g /L
size	Nanoscale	Namometers (nm)
t	Time	Seconds (s) / Hours (h)
U	Enzyme unit	μmol /min

Ψ	Volume	Liter (L) / cubic meter (m³)
W	Weight	Grams (g)
ϵ	Molar absorptivity	$\mu\text{M}^{-1} \text{ cm}^{-1}$
τ	Hydraulic retention time in the anode chamber	s

Gluconobacter sp.33 was used for production of alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (PQQ-ALDH). The growth of this bacteria was assayed in several fermentation MEDIA but only in YPM + GLY medium was obtained the specific growth rate of bacteria, ($\mu = 0.1187 \text{ h}^{-1}$) and proved to be the best medium for growth of bacteria, as well as for enzymes production. The enzyme activity of PQQ-ADH /ALDH were 8.00 and 11.42 U/mL, respectively and they are stable till 6 weeks when storage at 4 °C. The growth of *Gluconobacter sp.33* in YPM medium supplement with glycerol had already been studied however still no proved the induction of PQQ-ADH and PQQ-ALDH production. In this work glycerol proves induce enzyme biosynthesis and it is an important innovative point in this dissertation since this can lead to new developments and investigations in this field.

Studies of enzyme extraction were performed and parameters involved in cell permeabilization, extraction and purification of enzymes from supernatant were evaluated such as: the effect of sonication, storage, deoxycholate and lysozyme proportion on the loss of enzymatic activity. It is clear that occurred loss of enzymatic activity during sonication step. Also, it was proved the cells ensure more enzymatic activity when stored at – 20 °C, as well as the ideal proportion of deoxycholate and lysozyme is 0.5 %. In other hand, the extraction and purification of enzymes from supernatant of basal medium (BM) didn't show relevant values of enzymatic activity.

For electrochemical measurements was made a working electrode based on carbon felt, where it was immobilized cells and enzymes from supernatant through 0.5% of Glutaraldehyde. The cyclic voltammetry confirms the redox reaction that can occur through the PQQ-enzyme activity. The ethanol and glycerol oxidation occur electrons transfer according two mechanisms; Direct electron transfer (DET) and Mediated electron transfer MET. Only MET mechanisms presented relevant results, in terms of current density our working electrode with immobilized *Gluconobacter sp.33* cells achieved 131, 105 and 74 $\mu\text{A}/\text{cm}^2$, for cells with 10 mM ethanol, supernatant with 10 mM ethanol and cells with 10 mM glycerol. The chronoamperometric assay showed a linear increase of current as substrate concentration added from $3.93 \times 10^{-3} \text{ A}$ to $5.78 \times 10^{-3} \text{ A}$ and from $4.68 \times 10^{-5} \text{ A}$ to $4.15 \times 10^{-4} \text{ A}$ with ethanol and glycerol, respectively.

The aim of this dissertation is an assembly of BFC in order to generation power, by DET and MET mechanisms from a bioanode based on carbon felt, with immobilized cells using ethanol and glycerol as fuels. However, only MET mechanism have relevant results of power production. The maximal power density produced by surface area was 4.55×10^{-7} and $1.28 \times 10^{-7} \text{ W}/\text{cm}^2$ with ethanol and glycerol, respectively. The results of power production are too far from the expected, however this is challenge of Biofuel cells to reach in the future power production in same range than conventional fuel cells.

Keywords: *Gluconobacter sp.33*; PQQ-ADH / ALDH; Glycerol induction; Cyclic Voltammetry; Chronoamperometry; Biofuel Cells; Direct electron transfer (DET); Mediated electron transfer (MET); Power Generation; Power Generation by surface area.

Através da fermentação *Gluconobacter sp.33* foram produzidas álcool desidrogenase (PQQ-ADH) e aldeído desidrogenase(PQQ-ALDH). O crescimento desta bactéria foi testado em vários meios, contudo apenas foi relevante no meio YPM + GLY, no qual foi obtida a taxa específica de crescimento de $\mu = 0.1187 \text{ h}^{-1}$. Além do YPM +GLY provar ser o melhor meio para o crescimento da bactéria, o mesmo se comprovou relativamente à biossíntese das enzimas. O crescimento da *Gluconobacter sp.33* no meio YPM suplementado com glicerol já foi estudado, porém ainda não foi verificado que esta suplementação pode induzir a produção de PQQ-ADH e PQQ-ALDH. Este facto é provado neste trabalho pelo aumento dos níveis de atividade enzimática das duas enzimas. Isto é um ponto importante desta dissertação, uma vez que tem um carácter inovador e pode levar a novos desenvolvimentos e investigações neste campo. Relativamente às enzimas, estas mostraram níveis consideráveis de atividade enzimática, mais precisamente, as PQQ-ADH /ALDH têm 8.00 e 11.42 U/mL respetivamente. Além disso, ainda foi constatado que as enzimas são relativamente estáveis ao longo do tempo, isto é, mantiveram a sua atividade pelo menos seis semanas após a sua produção, quando armazenadas a 4°C.

Estudos de extração e purificação das enzimas a partir do sobrenadante foram efetuados. Para isso, foram verificadas várias etapas do processo de permeabilização celular, tais como: a sonicação, o armazenamento e a proporção da solução de deoxicolato, de forma a perceber como interferiam com a perda de atividade enzimática. Foi evidente a perda de atividade enzimática durante a sonicação e por isso foi retirado esta etapa do processo de permeabilização celular. Ainda, foi possível verificar que as células garantiam mais atividade quando armazenadas a -20°C , bem como que a proporção ideal para a permeabilização celular é de 0.5%. Por outro lado, a extração e purificação, das enzimas a partir do sobrenadante do meio basal (BM) não mostraram níveis relevantes de atividade enzimática.

Para as medidas eletroquímicas foi construído um eléctrodo de trabalho baseado em feltro de carbono, no qual foram imobilizadas as células e as enzimas, provenientes do sobrenadante, com solução de 0.5% de glutaraldeído. A voltametria cíclica foi realizada para observar e confirmar a reação redox que pode ocorrer através da atividade da enzima PQQ e de acordo com os dois mecanismos de transferência de electrões: transferência direta de electrões (DET) e a transferência mediada de electrões (MET), utilizando como substratos o etanol e o glicerol. Só o mecanismo MET apresentou resultados relevantes, em termos de densidade de corrente o nosso eléctrodo conseguiu produzir cerca de 131,105 and 74 $\mu\text{A}/\text{cm}^2$, para células com 10 mM etanol, sobrenadante com 10 mM de etanol e células com 10 mM de glicerol, respetivamente. Após isto, os ensaios de cronoamperometria mostraram um aumento linear da corrente à medida que a concentração de substrato foi aumentando, mais propriamente, de $3.93 \times 10^{-3} \text{ A}$ até $5.78 \times 10^{-3} \text{ A}$ e de $4.68 \times 10^{-5} \text{ A}$ to $4.15 \times 10^{-4} \text{ A}$, para as células com etanol e glicerol, respetivamente.

O principal objectivo desta dissertação foi a montagem e estudo de uma célula de biocombustível para produção de electricidade, nomeadamente através dos mecanismos de DET e MET, a partir de um bioânodo baseado em feltro de carbono com células imobilizadas de *Gluconobacter sp.33* e utilizando etanol e glicerol como combustíveis. No entanto, só o mecanismo MET teve resultados relevantes de produção de energia. A energia máxima produzida pela área de superfície foi de 4.55×10^{-7} e $1.28 \times 10^{-7} \text{ W}/\text{cm}^2$, para células com etanol e glicerol, respetivamente. Os resultados obtidos para a produção de energia ainda estão muito longe do que é esperado, contudo este é o desafio das células de biocombustível, alcançar no futuro a gama de geração de energia das células de combustível tradicionais.

Palavras-chave: *Gluconobacter sp.33*; PQQ-ADH / ALDH; Indução pelo glicerol; Voltametria cíclica; Cronoamperometria; Células de Biocombustível; Transferência direta de electrões (DET); Transferência mediada de electrões (MET); Geração de Energia; Geração de energia pela área de superfície.

CHAPTER 1: INTRODUCTION

1.1. MOTIVATION

Economic and environmental factors along with the human consumption pattern (which heavily relies on nonrenewable fuel sources) have called for “clean”, “green” and efficient energy production processes. The increasing energy demand associated with the rapid growth of the world population has engaged many research teams worldwide in developing viable processes to obtain efficient and sustainable energy. In this scenario, producing renewable energy may constitute a mean to relieve the worrying issue of global warming and provide new alternatives to the current energy consumption behavior [1]. In this context, Fuel Cells (FCs) concept was born and developed.

Different types of basic fuel cells exist, depending on the type of electrolyte and operation temperature. This technology offers considerable advantages over other processes, such as high conversion efficiency and generation of substantial power density. Although fuel cells yield good results, some factors limit their large-scale application: high cost and future scarcity of noble metal catalysts (e.g., platinum, employed as base catalyst in many fuel cell devices), issues regarding electrode passivation, and inability to oxidize some byproducts of the potential employed fuels [1-4]. Due to some of these advantages more recently was developed a new generation of FCs which use biocatalysts and named Biological Fuel Cells or only BioFuel Cells (BFCs).

In the broadest sense, biological fuel cells are defined as devices capable of directly transforming chemical to electrical energy via electrochemical reactions involving biochemical pathways of whole microorganisms or enzymes. Due to nature's versatility, BFCs in contrast with FCs are not limited to use of hydrogen or methanol as a fuel, and can derive electrical power from a wide range of organic substrates [2].

1.2. MAIN OBJECTIVE

The aim of this Master Dissertation developed along of the last months is the Production and characterization of pyrroquinoline quinone - dependent alcohol and aldehyde dehydrogenases (PQQ-ADH/ALDH) for assembly of Biofuel cells.

BFCs can be an important alternative sources of energy because, in nowadays the environmental problems are worrying the fossil fuels have been used the main and large source to produce energy. Moreover, the BFCs can be a strong alternative to implement in smaller or large devices with commercial interest, such as pacemakers, power banks, leads and others systems that don't require and consume great amount of energy.

Thus, based in the recent research and advances in this field, this work has the objective to test and use of PQQ-enzymes cascade to oxidize ethanol and glycerol, as renewable fuel sources. The assembly of biofuel cell in this work and the Bioanode will be based on carbon felt electrodes impregnated with immobilized *Gluconobacter sp.33* cells or PQQ-enzymes extracted from the cells.

1.3. DISSERTATION ORGANIZATION

The present dissertation is organized in seven chapters. The first chapter is denominated of *Introduction*, and it is where the motivation and main objective of this project are stated. In the second chapter, *State of the art*, is carried out an overview of the biofuel cells developed until nowadays, more specifically to enzymatic fuel cells, because our work is based in these biocatalysts, to produce electrical energy.

In the *Materials and Methods* chapter is mentioned the list of materials and all the methodologies used during this project as well the mathematics expressions to calculate relevant results. The Results and respective discussion are described in the Results and Discussion chapter. The last chapter, *Conclusions and Future Perspectives* summarizes the work developed, presenting the main conclusions based on the results obtained, but also suggestions of possible future work that can be useful for the continuation of present project. Finally, in *References* are provided all bibliography references used and in the *Annexes* are included all the supplementary results that can be auxiliary in interpretation of results and some experiments.

CHAPTER 2: STATE OF ART

2.1. BIOFUEL CELLS

The concept of using microbial organisms for electricity production was born in the end 19th century, when M. C. Potter noted that a culture of the bacterium *E. coli* produced electricity in half-cell studies employing platinum electrodes [1]. After discovering it, in few years the interest and advances in this area has been growing [1,2].

The biofuel cells (BFCs) are divided in three different groups: Microbial fuel cells (MFCs), when use as biocatalyst a microorganism, cells which use a primary fuel, usually an organic waste, and generate hydrogen, which is then used as a fuel within a conventional hydrogen/oxygen fuel cell; Enzymatic fuel cells (EFCs), which have a partial or pure enzyme to generate electricity directly from an organic fuel such as glucose; and Photochemical fuel cells (PHFCs) that use biological species to capture light and convert this into electrical energy, otherwise cells which combine the utilisation of photochemical active systems and biological moieties to harvest the energy from sunlight and convert this into electrical energy [2–5]. This work will give emphasis to BFCs, in particular, EFCs.

BFCs can be a strong alternative on the energy production in the future, because fossil fuels have been consumed exhaustively and ever more environmental problems are worrying [9]. The increasing energy demand associated with the rapid growth of the world population has involved authorities, governments, companies, and many research teams worldwide in developing viable processes to obtain efficient and sustainable energy [1]. The advantages of BFCs are vast, residing in the fact that they are “green” and clean sources to produce energy, mainly from the environmental point of view. In addition they are a silent technology [1].

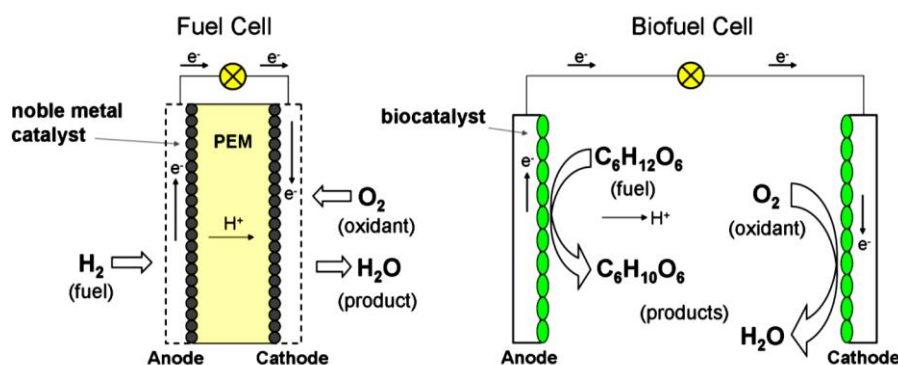


Figure 1: Schematic representation of conventional fuel cell, polymer electrolyte membrane (PEM) and enzymatic biofuel cell (EFC) [8].

The mechanism of these devices consists of a system that generate electrical energy from electrochemical reactions involving chemical species oxidation and reduction, as shown in Figure 1. In other words, traditional fuel cells use noble metal catalysts to generate electrons from fuel oxidation (typical fuels are hydrogen or small organic molecules such as methanol, ethanol, and glutaraldehyde, among others). After the oxidation step, an external circuit transfer the electrons to the cathode side where the electrons react with an oxidant molecule (usually oxygen), and generate electrical work as well as water and heat [2, 6-7].

The working principle of biofuel cells is the same as in conventional fuel cells, however the noble metal catalyst is substituted by a biocatalyst. It provides several benefits such as: the biocatalysts are inexpensive and their extended usage is expected to lower the cost of production, opposed to transition metal catalysts due to their limited availability. They are highly efficient systems exhibiting high turnover numbers, selectivity and activity under mild conditions (neutral pH and near-body temperature) [12]. The substrate specificity diminishes reactants cross-over, which theoretically enables a fuel cell design without a membrane. In addition, biocatalysts allow the utilization of more complex fuels (as their natural substrates abundant in nature), opposed to the relatively poor chemistry of hydrogen and methanol as typical fuels for conventional fuel cells [5, 7- 8].

The application of these systems covering several areas, as biomedical systems, such as cardiac pacemakers where the EFCs was using to provide energy into this system, a glucose sensor for diabetics, neurostimulators, hearing and vision devices, drug pumps, bladder-control valves, etc., all of biomedical accessories that would take advantage of a small implantable power source based in EFCs [7,9]. However, some of these systems cannot compete directly with conventional batteries, for example, in the case of pacemakers due to short operational life and in the case of neurostimulators, due to limited power output [7, 10]. Concerning to the field of applications MFCs these have implementation in the treatment of waste water courses, which consumes a wide range of organic wastes (e.g. corn husks, whey or noxious waste such as animal or human sewage). Still, fermentation processes can consume waste substrates reducing their environmental impact and simultaneously producing ethanol or hydrogen, which can then be used to generation of power in a conventional H_2/O_2 or ethanol/ O_2 fuel cell [8].

The future of BFCs involves new practical applications and the resolution of some of their limitations. One of the primary challenges at hand is increased biocatalytic power density, as well as, establishing a better electrical communication between the protein and the electrode surface, and by the limited stability of the biocatalyst-electrode assembly [8, 9].

2.2. ENZYMATIC FUEL CELLS

Based in references, the first attempt of assembly EFCs occurred in 1964. Yahire et al. assembly the first system using glucose oxidase and glucose as a fuel and oxygen in air as an oxidant [14].

Enzymatic Fuel Cells can be described as a system, that use isolated enzymes, essentially produced and extracted from microorganisms, to catalytically oxidized a specific fuel at the anode

compartment and then, reduction of the oxidant, usually oxygen, at the cathode compartment [15]. A choice of the enzyme is an important factor, because they have to be able to oxidize the fuel under relatively mild conditions (neutral pH, ambient temperature) compared to conventional fuel cells [6]. A general process of these systems is exemplified in Figure 2.

The structure of EFCs consists of two compartments: anode and cathode wherein single enzymes (or enzyme cascades) allows to have defined reaction pathways on the electrode surface to produce a power electricity [8-12]. Much attention has focused on proof of this concept and production of some prototype EFCs, because of advantages that the use of biologically derived catalysts can offer.

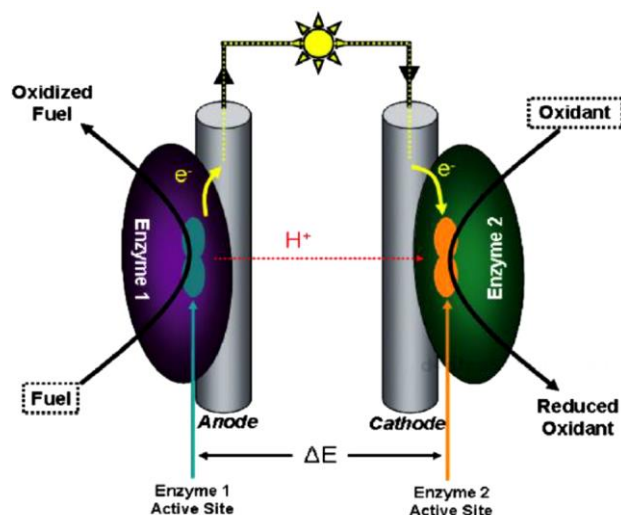


Figure 2: Representation of example the redox processes in a membrane of the Enzymatic Fuel Cells (EFCs) [9].

Enzymes are attractive to use in EFCs as electron shuttles due to their high substrate specificity, i.e. being very selective in terms of the fuel or oxidant that is oxidized or reduced, making half-cell separation, i.e., usage of membranes unnecessary. Also, enzyme production is relatively inexpensive, enabling their use in non-generic applications [2, 5, 7, 13-14].

The ability of enzymes to utilize biologically derived fuels, such as e.g. glucose, fructose, lactose, ascorbate, dopamine, and alcohols, along with ubiquitous O_2 as the biooxidant, makes the use of enzyme based BFCs very attractive for an array of applications, especially as electric power sources in implantable devices in living organisms, such as a pacemaker and a glycoses sensor to a diabetes. The enzymes themselves, as well as their reaction products, can also be considered as relatively safe compared to non-biogenous catalysts; an important consideration indeed, in an implantable situation [2, 5, 7, 13-14].

2.2.1. ENZYMATIC FUEL CELLS – OVERVIEW

An overview of some typical EFCs configurations based in literature is presented in Table 1. It is difficult to compare directly the performance of these BFCs due to the variety of experimental conditions used. Nevertheless, it is presented some parameters in order to provide an idea of their typical values and efficiency.

Table 1: Examples of Enzymatic Fuel Cells developed until nowadays. Adapted from [11].

Fuel Oxidant	Enzymes Anode Cathode	Mediators Anode Cathode	Power Density ($\mu\text{W. cm}^{-2}$)	Fuel Concentration (mM)	Reference
Glucose O ₂	GO _x Laccase	Ferrocene -	15,8	10	[17]
Glucose O ₂	GO _x BOD	HQS ABTS	42	10	[18]
Glucose O ₂	GO _x Laccase	TTF ABST	7	15	[19]
Glucose O ₂	GO _x Laccase	Os polymer Os polymer	137 - 350	15	[19–21]
Glucose O ₂	GO _x BOD	Os polymer Os polymer	50 - 480	15	[22–24]
Glucose H ₂ O ₂	GO _x MP-11	PQQ -	160	1	[26]
Glucose H ₂ O ₂	GO _x HRP	Ferrocene Ferrocene	0,15	1	[27]
Glucose O ₂	GDH Laccase	Azine dyes -	58 – 38,7	45 - 60	[27–30]
Glucose O ₂	GDH BOD	Azine dyes -	52 – 53,9	40	[31-32]
Glucose O ₂	GDH BOD	VK ₃ Ferricyanide	1450	400	[34]
Glucose O ₂	CDH -	Os polymer -	157	100	[35]
Lactose O ₂	CDH Laccase	Os polymer Os polymer	1,9	34	[36]
Fructose O ₂	FDH Laccase	-	850	200	[37]
Fructose O ₂	FDH BOD	-	126	200	[38]

These systems have been improved along the time, mainly, to provide a better electron transference (see next chapter), a complete oxidation of fuels in order to improve global yield of reactions and the-capacity to produce of energy [2, 8-9].

Some enzymatic fuel cells developed until nowadays based on enzymes that will be studied in this work, PQQ - dependent alcohol dehydrogenase (PQQ-ADH) and PQQ - dependent aldehyde dehydrogenase (PQQ-ALDH) are shown in Table 2.

Table 2: Examples of Enzymatic Fuel Cells developed until nowadays, based in enzymes that will be studied in this project.

Fuel Oxidant	Enzymes		Power Density ($\mu\text{W} \cdot \text{cm}^{-2}$)	Fuel Concentration (mM)	Reference
	Anode Cathode	Mediators Anode Cathode			
Glucose O_2	GO_x CO_x cytochrome c	PQQ -	5	1	[39]
Methanol O_2	ADH -	Benzylviologen -	670	100	[40]
Ethanol H_2O_2	PQQ-ADH AO_x MP -8	-	1,5	25	[41]
Ethanol O_2	ADH ALDH BOD	Poly(methylenegreen) $\text{Ru}(\text{bpy})_3$	460	1	[42]
Glycerol O_2	PQQ-ADH PQQ-ALDDH - OO_x	Poly(methylenegreen) -	1320	100	[43]
Pyruvate O_2	PDH -	Poly(methylenegreen) -	930	100	[44]

2.3. Enzymatic Fuel Cells – Electrons Transference

Classification of the electron transfer between enzymes and electrode surfaces depends on the way electrons move from the enzyme catalytic site to the electrode surface. Therefore, there are two main processes: direct processes are known as direct electron transfer (DET) and processes requiring the assistance of a mediator molecule are designated mediated electron transfer (MET), observed in Figures 3 and 4 [2-3, 8-9, 14-15].

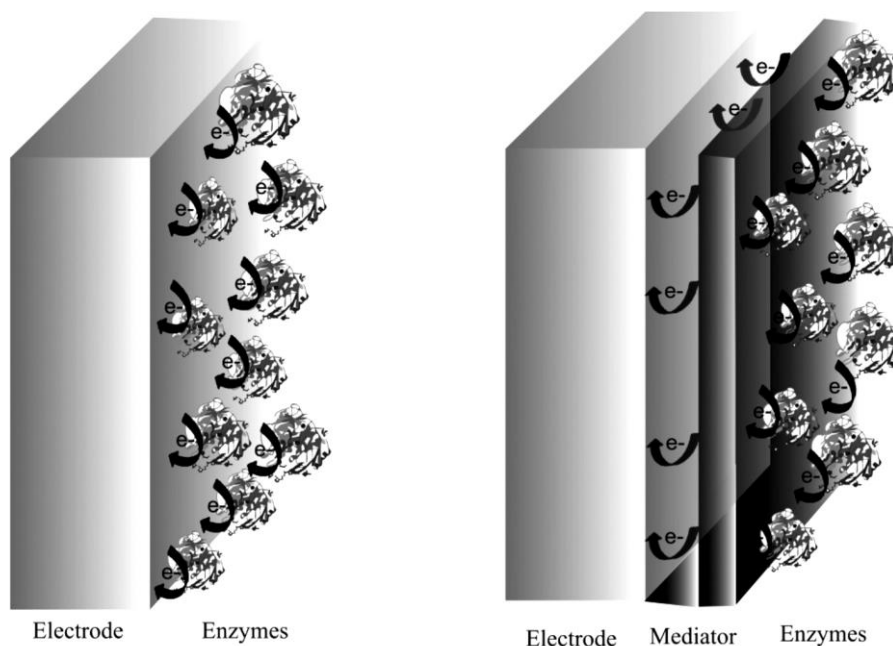


Figure 3: Types of electron transfer processes between enzymes and electrode surfaces in EFCs. Direct processes are known as direct electron transfer (DET), left, and processes requiring the assistance of a mediator molecule are designated mediated electron transfer (MET), right.

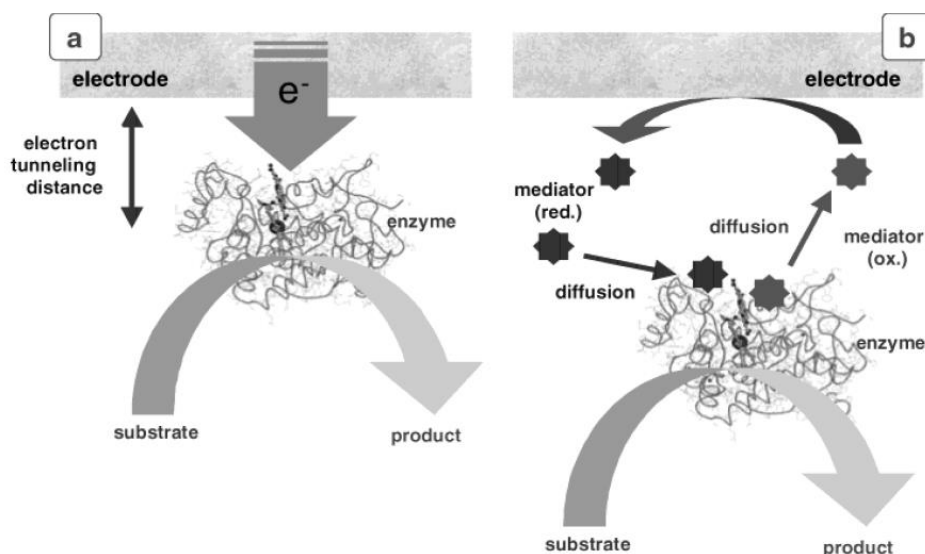


Figure 4: Electron-transfer mechanisms. (a) Direct electron transfer from electrode surface to the active site of an enzyme. (b) Electron transference via redox mediator [12].

According to EFCs electrical communication, i.e. transferences of electrons, it can be divided into three different groups: The first group contains the redox centre located in a peripheral area of the enzyme, so it can directly transfer electrons to or accept electrons from an electrode surface. Here, it is included the PQQ-dependent dehydrogenase enzymes. The second group bears a weakly bound cofactor (NAD^+ or $NADP^+$) that acts as a mediator at the redox center. This species can diffuse to the electrode surface and carry the electrons from the enzymatic catalysis. The third group involves enzymes with a strongly bound redox center, normally located inside the protein shell [2-3, 8-9, 14-15].

2.3.1. DIRECT ELECTRON TRANSFER (DET)

In a DET, the enzymatic and electrode reactions are characterized by direct electron transfer, without mediators, as for example, $FAD^+/FADH$. In other words, the electrons are transferred directly from the electrode to the substrate molecule (or vice versa) via the active site of the enzyme. In this case, the distance between active site of enzyme and electrode surface is an important factor [2, 9, 17–19].

Therefore, in the DET mechanism, the enzymatic catalysis and the electrochemical reaction are not separate reactions, but an integrated process where the electron functions as a second substrate. The tunnelling mechanism of the electrons in a DET enzymatic system will depend on the enzyme structure, the redox centre location, the enzyme orientation on the electrode surface, and the distance of the electron transfer. Hence, a good electron transfer rate between enzymes and electrodes will only be possible if all these conditions are met. One great advantage of this type of electron transfer process is that it eliminates the issues related to use of mediator species, avoiding performance losses that may arise from the potential difference between the enzymes and the mediator species [18-21].

Direct electron transfer has been reported for a wide range of enzymes, such as, laccase, lactate dehydrogenase, peroxidase, hydrogenase, p-cresolmethylhydroxylase, methylamine dehydrogenase, succinate dehydrogenase, fumarate reductase, D-fructose dehydrogenase, alcohol dehydrogenase (will be used in future work), and D-gluconate dehydrogenase [9,19]. However, several challenges need to be overcome to achieve significant rates of direct electron transfer, leading to appreciable current densities, between active sites and solid electrode surfaces [2].

2.3.2. MEDIATED ELECTRON TRANSFER (MET)

As an alternative to DET, small, artificial substrate/co-substrate electro active molecules (mediators) can be used to shuttle electrons between the enzymes and the electrode, in a process referred to as mediated electron transfer (MET) [3, 9,12].

In this case, the enzyme catalyses the oxidation or reduction of the redox mediator. The reverse transformation (regeneration) of the mediator occurs on the electrode surface. The major characteristics of mediator-assisted electron transfer are that: the mediator acts as a cosubstrate for the enzymatic reaction and the electrochemical transformation of the mediator on the electrode has to be reversible. In these systems, the catalytic process involves enzymatic transformations of both the first substrate (fuel or oxidant) and the second substrate (mediator). The mediator is regenerated at the electrode surface, preferably at low voltage. The enzymatic reaction and the electrode reaction can be considered occur separate but at same time [2, 9, 16–18, 22].

In MET, the thermodynamic redox potentials of mediators now dictate the maximum EFCs cell voltage. Thus, a redox potential which is more positive for oxidative biocatalysis (at anode) and more negative for reductive biocatalysis (at cathode) is required in order to provide a driving force for electron transfer between enzyme active site and mediator, both contributing to cell voltage in an EFC. A major challenge in MET is therefore to achieve the best compromise between driving force and current, in order to maximise power output [2, 21].

Although MET-based bioelectrodes require additional species during biofuel cell preparation, MET is generally preferred over DET, because it can generate higher output power with often large orders of magnitude than the direct mechanism. The possibility of using commercially available enzymes, such as many NAD⁺ - dependent alcohol dehydrogenases and FAD⁺ - dependent glucose oxidase and dehydrogenases, is another advantage of this methodology. The mediator molecules can be either anchored onto the electrode surface (e.g., in the form of a polymeric film), free in solution, or even linked to the structure of the enzyme; it withdraws the electrons generated during the enzymatic catalysis and transports them to the electrode surface [7, 23–25].

These mediator species must be able to efficiently perform the enzyme/electrode connection, to rapidly react with the reduced form of the enzyme, and be soluble in both its reduced and oxidized forms, so that it can diffuse to the electrode/enzyme fast. Moreover, they should be non-toxic, stable, and biocompatible [2-3, 24-25].

2.4. FUELS, OXIDANTS AND ENZYMES USED IN EFCs

2.4.1. FUELS MOST USED IN EFCs

The most fuels used in EFCs, included a variety of sugars, and low aliphatic alcohols, that can be used due a vast nature of employed catalysts in this type of devices [11].

The most common fuel for EFCs is undoubtedly the glucose. This due to its high abundance in nature and essential role in human metabolism. Glucose is an important metabolic intermediate and a source of energy for a variety of living organisms. It is a carbohydrate and it is involved in the glycolysis metabolic pathway, where it is oxidized to pyruvate, which further enters the citric acid cycle. Eventually, after series of chemical transformations with a release of energy, glucose is broken down to CO₂ and water [8, 16–18].

Other sugars that have been employed as fuels are fructose, which is a structural isomer of glucose as well as some disaccharides as lactose and cellobiose, however without relevant results [11].

Other fuels that have been used in EFCs, such as, methanol, ethanol and glycerol. Methanol has been already identified as one of the best fuels in conventional direct fuel cells and the ethanol is already commercially available for combustion engines. Glycerol, that will be used in our EFCs, is an attractive fuel due to its high energy density, low vapour pressure and low toxicity opposed to the latter alcohols [38–41]. All three alcohols are considered as renewable fuels, so they can be produced from renewable biomass, which may be an advantage for their use. The main fuels and the respective enzymes used for their oxidation are listed in Table 3.

Table 3: Fuels and enzymes most used in EFCs. Adapted from [11].

Fuel	Enzyme	Co-factor	Half-Cell Reaction	Natural Acceptor
Glucose	Glucose oxidase EC 1.1.3.4	FAD	$\text{Glucose} \rightarrow \text{Glucono-1,5-lactone} + 2\text{H}^+ + 2\text{e}^-$	O_2
	Glucose dehydrogenase EC 1.1.1.47	NAD		NAD
	Glucose dehydrogenase EC 1.1.5.2	PQQ, heme		Quinone
	Gellobiose dehydrogenase EC 1.1.99.18	FAD, heme		Acceptor
Methanol	Alcohol dehydrogenase EC 1.1.1.1	NAD	$\text{Alcohol} \rightarrow \text{Aldehyde} + 2\text{H}^+ + 2\text{e}^-$	NAD
	Aldehyde dehydrogenase EC 1.2.1.5	NAD	$\text{Aldehyde} + \text{H}_2\text{O} \rightarrow \text{Acid} + 2\text{H}^+ + 2\text{e}^-$	NAD
	Formate dehydrogenase EC 1.2.1.2	NAD	$\text{Formate} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$	NAD
	Alcohol dehydrogenase EC 1.1.99.8	PQQ, heme	$\text{Alcohol} \rightarrow \text{Aldehyde} + 2\text{H}^+ + 2\text{e}^-$	Acceptor
Ethanol	Alcohol dehydrogenase EC 1.1.1.1	NAD	$\text{Alcohol} \rightarrow \text{Aldehyde} + 2\text{H}^+ + 2\text{e}^-$	NAD
	Aldehyde dehydrogenase EC 1.2.1.5	NAD	$\text{Aldehyde} + \text{H}_2\text{O} \rightarrow \text{Acid} + 2\text{H}^+ + 2\text{e}^-$	NAD
	Alcohol dehydrogenase EC 1.1.99.8	PQQ, heme	$\text{Alcohol} \rightarrow \text{Aldehyde} + 2\text{H}^+ + 2\text{e}^-$	Acceptor
Glycerol	Alcohol dehydrogenase*	PQQ, heme	$\text{Alcohol} \rightarrow \text{Aldehyde} + 2\text{H}^+ + 2\text{e}^-$	-
	Aldehyde dehydrogenase*	PQQ, heme	$\text{Aldehyde} + \text{H}_2\text{O} \rightarrow \text{Acid} + 2\text{H}^+ + 2\text{e}^-$	-
	Oxalate oxidase* EC 1.2.3.4	FAD, Mn	$\text{Oxalate} \rightarrow 2\text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$	O_2

2.4.2. PQQ-ADH / PQQ-ALDH AND GLYCEROL - OUR BFCs

Glycerol (1,2,3-propanetriol or glycerine), an organic molecule is obtained as a by-product (10 % in weight) in manufacturing biodiesel fuel by transesterification of seed oils with methanol [55]. Glycerol ($C_3H_8O_3$) have a density value of the $1,261 \text{ g.cm}^{-3}$, molecular mass $92,009 \text{ g.mol}^{-1}$ and $1,5 \text{ Pa.s}$ of viscosity. The melting and boiling points are $18,2 \text{ }^\circ\text{C}$ and $290 \text{ }^\circ\text{C}$, respectively [56].

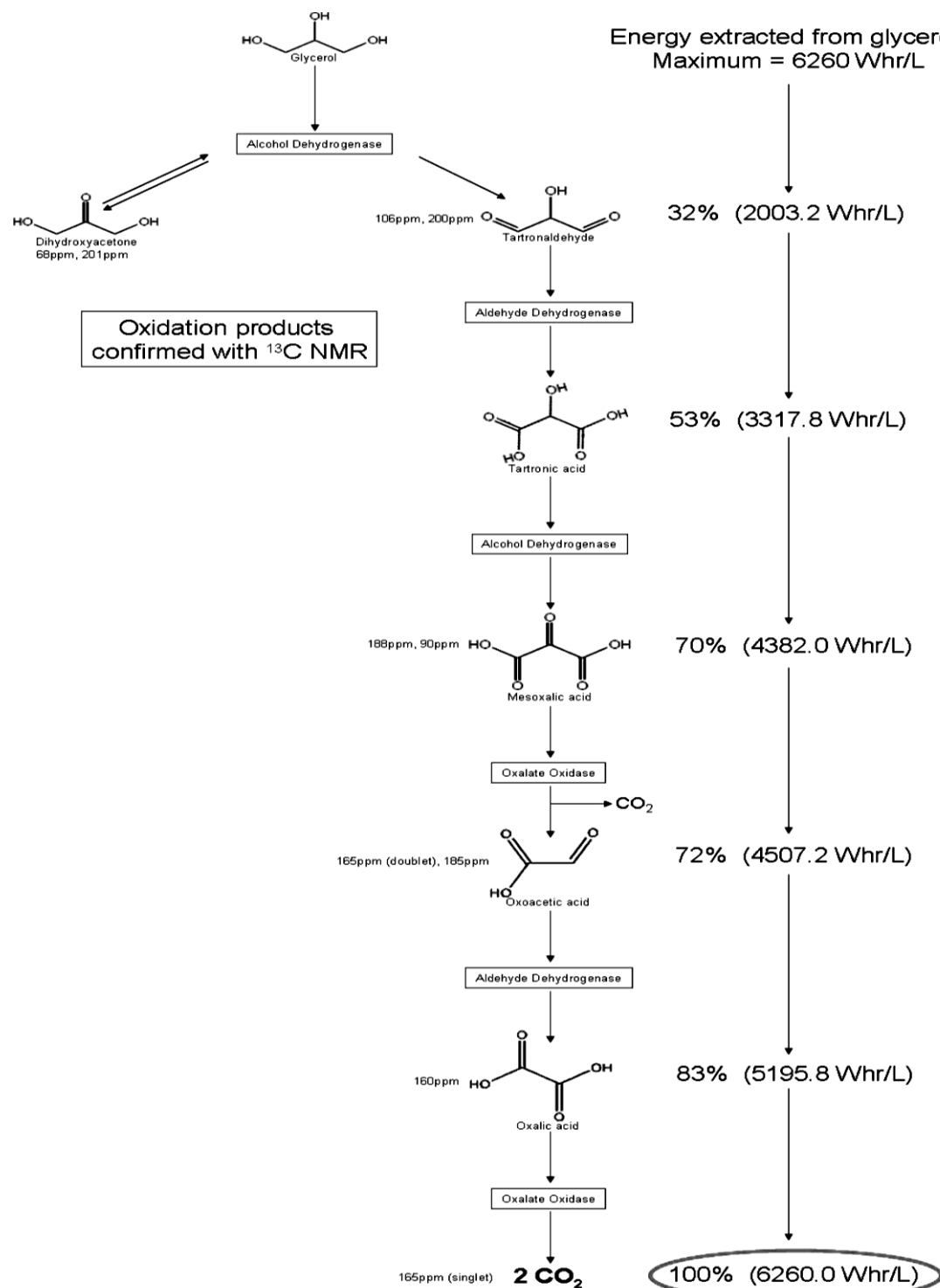


Figure 5: Enzyme cascade reaction of the complete oxidation of glycerol, that will be used in this work [43].

Glycerol will be used in this work in a EFCs that utilizes a two PQQ-enzyme cascade of *Gluconobacter* sp. 33 on the anode can accomplish until 70 % oxidation of glycerol. The enzymes utilized are PQQ- dependent alcohol dehydrogenase (PQQ-ADH) and PQQ dependent aldehyde dehydrogenase (PQQ-ALDH), both produced or extracted from *Gluconobacter* sp.33. With the use of heterogeneous oxalate oxidase eventually complete oxidation could be achieved [43].

PQQ-ADH and PQQ-ALDH can oxidise glycerol up to five times leaving mesoxalic acid as a by-product. Additionally, it employed the oxalate oxidase to finish the oxidation of the mesoxalic acid to CO₂. By using oxalate oxidase, the level of oxidation of the fuel is increased from 70% (4,382 W h L⁻¹) to 100% (6,260 W h L⁻¹) as shown in Figure 5 [43].

PQQ-ADH (Pyrroquinoline quinone -Dependent Alcohol Dehydrogenase) consist of subunits I, II, and III, except for PQQ-ADHs purified from *Gluconacetobacter* species, which consist of only the subunits I and II. The subunit I is approx. 80 kDa in the molecular size and contains pyrroquinoline quinone (PQQ). This subunit functions as the catalytic site for ethanol oxidation. The subunit II is approx. 50 kDa in the molecular size and as function the electron mediator from the subunit I to the membranous [57].

As a PQQ-ADH, the PQQ-ALDH (Pyrroquinoline quinone - Dependent Aldehyde Dehydrogenase) is a heterodimer comprising two subunits of 79.7 and 50 kDa in the molecular size, respectively [58]. The smaller subunit bears three cytochromes c. Aliphatic aldehydes, but not formaldehyde, were suitable substrates. Using ferricyanide as electron acceptor, the enzyme shows a optimum pH of 3.5 that shifted to pH 7.0 when phenazine methosulphate (PMS) plus 2,6-dichlorophenolindophenol (DCPIP) were the electron acceptors [58].

PQQ -ADH and PQQ-ALDH, which are located in the cytoplasmic membrane, transfer electrons to ubiquinone Q10. PQQ-ADH is a periplasmic quinoxinoprotein-cytochrome c complex and catalyses the first step of ethanol oxidation by transferring electrons to Q10 and producing acetaldehyde, which usually is the substrate for another enzyme (PQQ-ALDH), and converted to acetic acid during the second step of ethanol fermentation [58-59].

2.4.3. OXIDANTS MOST USED IN EFCs

The most widely referred oxidant in use of EFCs is oxygen and there are only few reports of other compounds. Oxygen is the typical oxidant in conventional fuel cells, where it is used in the form of pure gas or air. In the case of enzymatic fuel cells, oxygen is usually used dissolved in aqueous electrolyte and in absence of enzymes, whereby its low water solubility raises additional mass transport problems, however there are also reports of EFCs utilizing gas phase oxygen or air [8,17,41].

The respective half-cell reactions and enzymes of some EFCs used for the bioelectrochemical reduction are listed in Table 4.

Table 4: Oxidants and enzymes most used in the cathodic chamber of EFCs. Adapted from [11]

Oxidant	Enzyme	Metal Co-factor	Half-Cell Reaction
Oxygen	Laccase, EC 1.10.3.2	Cu	$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$
	Bilirubin Oxidase, EC 1.3.3.5	Cu	
	Cytochrome Oxidase, EC 1.9.3.1	Cu, Fe heme	
	Cytochrome c, -	Fe heme	
	(Absence of enzyme)	-	
Hydrogen Peroxidase	Microperoxidase-11, -	Fe heme	$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$
	Horseradish Peroxidase, EC 1.11.1.7	Fe heme	
	(Glucose, GOx) Microperoxidase-8, -	Fe heme	
Cumene Peroxidase	Microperoxidase-11, -	Fe heme	$C_9H_{12}O_2 + 2H^+ + 2e^- \rightarrow C_9H_{12}O + H_2O$

Other oxidants has been rarely employed, such as hydrogen peroxide, however the highly reactive oxygen species has strong oxidizing properties and can be harmful to biological components, as the enzymes [26]. There is also one report in the literature, where cumene hydroperoxide has been used as an oxidant based on two immiscible solvents [60].

2.4.4. ENZYMES MOST USED IN EFCs

Redox enzymes usually consist of an apoenzyme (the protein component of an enzyme) and at least one coenzyme (a small nonproteinaceous electroactive molecule). The presence of the coenzyme is essential for electron transfer between the enzyme and the substrate, which in EFCs can be also involved to carrier the electrons to the electrode surface, as seen in chapter 2. The coenzyme can be either tightly bound within the enzyme structure (coenzyme) or released from the enzyme's active site during the reaction (cofactor). Common coenzymes include flavin adenine dinucleotide (FAD) and pyrroloquinoline quinone (PQQ), and in cofactors nicotinamide adenine dinucleotide (NAD) [15].

The commonly reported anodic systems use only one enzyme, which catalyzes partial oxidation of the fuel. The redox process is associated with the breakage of one chemical bond, which limits the number of electrons that can be gained from the anodic reaction. In this context, recent advances shown that the employment of enzyme cascades, which allow better utilization of the chemical energy of the fuel and inclusively to complete oxidation to CO₂. This cascade of bioelectrochemical transformations on the electrode surface increases the total current density delivered by the fuel cell as the result of electrons from every single reaction [8, 38–41].

The most enzymes used in EFCs are listed in table 2 and some of these in table 3, and as expected the glucose oxidase is the most enzyme utilized in these systems, as glucose is most fuel used.

2.5. ARCHITECTURE AND MATERIALS USED IN EFCs

2.5.1. DESIGN AND ARCHITECTURE OF EFCs

The configuration of EFCs is very similar than of MFCs, consist essential in two chambers (cathode and anode) separated by selective membrane or set of membranes, in which the movement of electrons originates the production of electrical energy.

Enzymatic fuel cells are still at level of proof-of-concept, as such, still there are few EFCs designs. Thus, based in literature are reported four principal designs: microfluidic enzymatic fuel cells, concentric biofuel cell, enzymatic fuel cell with air breathing cathode, and the typical assembly of MFCs, with 2 compartments.

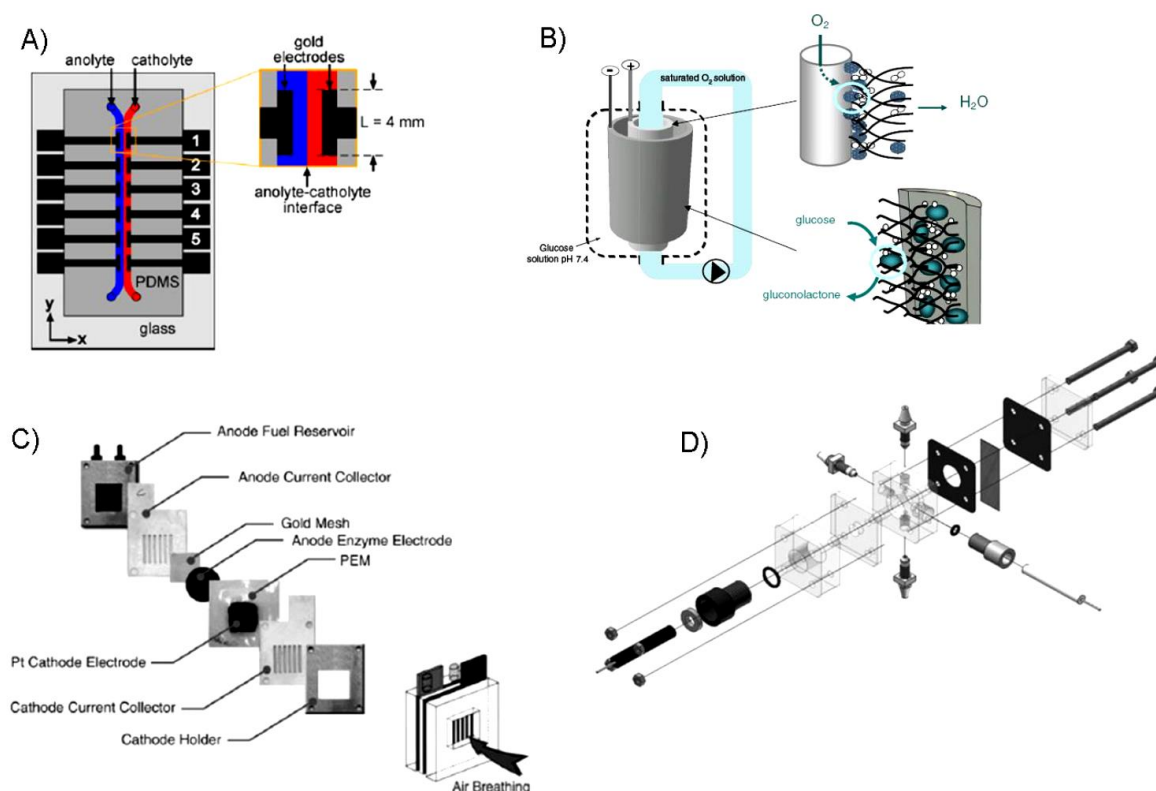


Figure 6: Representation of (A) microfluidic biofuel cell device; (B) concentric biofuel cell; (C) miniature biofuel cell consisting of an enzymatic anode and an air-breathing cathode; (D) standardized modular stack cell platform [11].

Microfluidic biofuel cells, usually employ diffusional enzyme and mediator, dissolved in the electrolyte solution flowing through the microchannel (Figure 6A). The typically low flow rates and micrometer-range dimensions define laminar flow conditions and the resulting lack of convective mixing allows the elimination of a physical membrane to separate the anolyte and catholyte flows [61].

The construction of concentric enzymatic fuel cells consists in two carbon tubular electrodes, placed in the same electrolyte, where at the cathode tube was inserted inside of the anode tube [18]. Oxygen saturated solution was supplied to the inner of the cathode and diffused to its outer side, where it took part in the bioelectrochemical conversion as can be seen in Figure 6B.

The low solubility of oxygen in aqueous electrolytes was already addressed and represented one important limitation in these devices. This limitation has motivated the employment of oxygen in gas-phase. Thus, the design of this structure without conventional cathodic chamber allows the reduction occur directly with O₂ from the air (as seen in Figure 6C) [62].

Due to the difficulty in comparing and interpreting results from different laboratories has motivated the development of a modular stack cell prototype as a standardized testing platform for enzymatic fuel cells (Figure 6 D). The proposed stack cell design is flexible and can be used for various electrochemical experiments. This approach allows for better controlling of experimental conditions [52].

2.5.2. MATERIALS

In this section will be presented some materials and their function in EFCs, in particular, those one that will be used in assembly the EFC of this work.

Materials – Carbon Felt

Carbon felt (CF) is a microelectrode ensemble of micro carbon fiber (ca. 7 μm diameter), and possesses a random three-dimensional structure with high porosity (>90%). The CF has high surface area (estimated to be 0.3–30 m^2/g) and shows a high conductivity and excellent electrolytic efficiency. In addition, porous structure of the CF causes very low diffusion barrier against the solution flow. On the basis of these characteristics, reported recently that the CF is useful as a Working Electrode (WE) unit of electrochemical flow-through detector [63].



Figure 7: Carbon Felt.

Nafion Membrane

Nafion is generated by copolymerization of a perfluorinated vinyl ether co-monomer with tetrafluoroethylene (TFE). The greatest interest in Nafion in recent years derives from its consideration as a proton conducting membrane in fuel cells [64].

It is clear that the tuning of these materials for optimum performance requires a detailed knowledge of chemical microstructure and nanoscale morphology. In particular, proton conductivity, water management, relative affinity of methanol and water in direct methanol fuel cells, hydration stability at high temperatures, electro-osmotic drag, and mechanical, thermal, and oxidative stability are

important properties that must be controlled in the rational design of these membranes. This is a challenge for Nafion materials in which the possible chemical variations are rather limited. And, of course, all of these objectives must be achieved while maintaining low cost. While a number of alternate polymer membranes, including nonfluorinated types, have been developed, Nafion is still considered the benchmark material against which most results are compared [64].

Ion Jelly

Ion Jelly allows to produce polymeric films with good electro-conductive properties, which are used to improve electron transference in the EFC of this work. This polymeric layer can be deposited on nafion membrane or carbon derivative material (e.g. paper or felt) [65]. The film is a solid, transparent and electro-conductive material prepared by combining a biopolymer, gelatine, and one ionic liquid, 1-butyl-3-methylimidazolium dicyanamide (BMIMDCA) (>98%) [66].

This electro-conductive film was elaborate during the preliminary results; it can be observed in this section as well as the procedure to obtain it.

2.6. DETERMINATION OF POWER GENERATION IN EFCs

2.6.1. CALCULATING POWER

For any fuel cell the power output, the voltage across the external resistor or load in an EFCs can be measured using a multimeter. Voltage measurements are convert to current values using Ohm's law (Equation 1) [7, 63]:

$$V = I R \quad (\text{Equation 1})$$

Where, V is voltage (volts); I is current (ampere) and R is resistance in (ohms). The power output from a EFCs is obtained by equation 2:

$$P = V \int I \quad (\text{Equation 2})$$

Where P is power (watt -W). And, if the current (I) is constant, the power is calculated by $P = VI$. And then power output is expressed in terms of the calculated current as:

$$P = I^2 R \quad (\text{Equation 3})$$

2.6.2. POWER DENSITY BY SURFACE AREA

Knowing how much power is generated by an EFCs it does not describe how efficiently that power is generated by the specific system architecture [7, 64 - 65]. Thus, it is common to normalize

power production by the surface area of the anode or in alternative by volume of anodic chamber, A and V respectively, so that the power density produced by the EFC is:

$$P_{DA} = \frac{I^2 R}{A_A} \quad (\text{Equation 4})$$

Where, P_{DA} is power density on area basics in W / m² and A_A is anode surface area in m². The power density may be calculated based on total or net anode chamber volume as follows:

$$P_{DV} = \frac{I^2 R}{V} \quad (\text{Equation 5})$$

Where P_{DV} is power density on volume basis in W / m³ and V is anode chamber in m³.

2.6.3. EFFICIENCY OF EFCs

The efficiency of an EFCs is often expressed as the Coulombic efficiency. The Coulombic efficiency is calculated in two ways, as in MFCs [64-65].

In the first, E_C is expressed as the ratio of coulombs transferred from the substrate to anode in relation to the theoretical maximum coulombs that would be produced if all of the substrate was oxidized. The coulombs of energy produced (C_P) and transferred to the anode is calculated for batch reactors by integrating the area under a curve of current versus time. Commonly, this is calculated simply as shown in equation 6:

$$C_P = I t \quad (\text{Equation 6})$$

Where t is time of stable voltage output in seconds (s). For continuous flow reactors, the C_P , produced at steady state is calculated as:

$$C_P = I \tau \quad (\text{Equation 7})$$

Where τ is hydraulic retention time in the anode chamber in seconds (s).

The theoretical maximum coulombs (C_{max}) that may be transferred from a specific substrate is calculated as:

$$C_{max} = \frac{F b S V}{M W} \quad (\text{Equation 8})$$

Where, F is Faraday's constant (96.485 C/mol of electrons); b is mol of electrons available for removal per mol of substrate; S is substrate concentration in g /L and MW is molecular of the substrate. The Coulombic efficiency (E_C) is then calculated according equation 9:

$$E_C = \frac{C_P}{C_{max}} \times 100\% \quad (\text{Equation 9})$$

2.6.4. POWER DENSITY AS FUNCTION OF SUBSTRATE

Power produced from an EFCs is a function of substrate concentration, as occur in a MFCs. The response can be modelled using Monod (Michaelis- Menton) relationship, as it can be observed in equation 10 [64,65].

$$P = \frac{P_{max} \times S}{(K_s + S)} \quad (\text{Equation 10})$$

Where, P represent power density in (mW/m²), P_{max}, maximum power produced in (mW/m²), S the substrate concentration in (mg/L) and K_s, half-saturation constant for substrate in (mg/L).

2.7. BIOFUEL CELLS - CHALLENGES

A few years ago, anyone could say that the applicability of the enzymatic biofuel cell as an alternative energy source was questionable or a dream that would be difficult to come true. Nowadays, although metallic-based fuel cells still stand out in terms of research and power output, the use of enzymes to efficiently convert energy from chemical substrates to electricity has increased [10].

These systems still need to meet the requirements of practical commercial application, though. Significant improvements in terms of enzyme immobilization, power density, stability, cost of the employed materials, and issues related to the electron transfer between enzymes and electrode surfaces still need to be achieved.

In particular, with enzymes, the lifetime of the biocatalysts, currently of the order of a month in favourable cases, needs to be increased to the order of at least a year to find commercial favour [15].

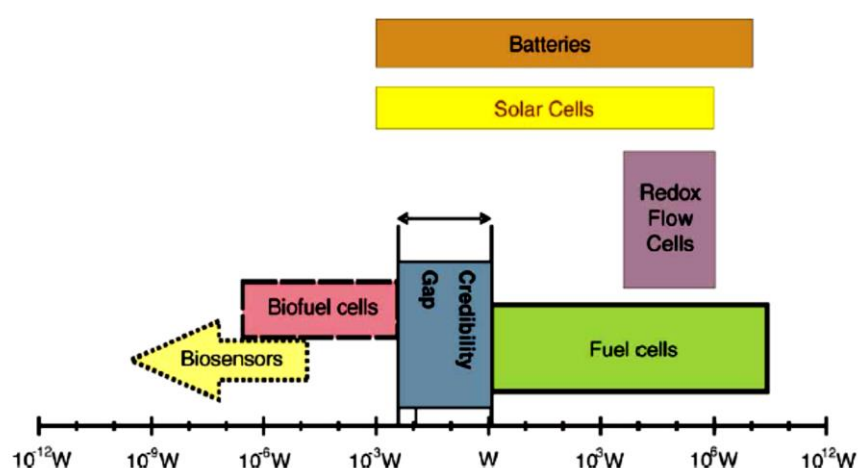


Figure 8: Approximate power output ranges of biosensors, biofuel cells and inorganic fuel cells, illustrating the scale of the credibility gap challenging the biofuel cell researcher [10].

Unfortunately, wide-scale comparison between devices (anodes, cathodes, assemblies), in terms of operating conditions such as performance of reference values, and stability benchmarks, is hampered by a lack of conformity to standardized procedures for testing and reporting of data. In addition, there is scope for greater understanding of the role that each component plays in an integrated system to provide for efficient EFCs, by comparison of performance to models, to enable identification of limiting factors in reaction kinetics, mass transport, and system design [3- 8, 12].

However, the advances have been achieved, as it reported in past studies, such as power output may be improved through use of optimized biocatalyst (and mediator if appropriate) on three-dimensional surfaces and deeper oxidation of fuels using multiple enzymes and enzymes cascades [43].

The principal challenge of biofuel cells is achieving the same power rate that other conventional fuel cells, in order to compete with them, as can be observed in Figure 8.

CHAPTER 3: MATERIALS AND METHODS

3.1. MATERIALS

For fermentation of *Gluconobacter sp.* 33 were used several media with different nutrients. They were purchased from several companies, such as: Yeast Extract Power (HIMEDIA), D-Mannitol (> 98%) (AppliChem), Bacteriological Peptone (Oxoid), Ammonium Phosphate (> 98%) (Panreac), D-Glucose anhydrous and Glycerol (> 99%) from Fisher Scientific, UK, Magnesium Sulfate (> 99%) and Calcium Carbonate (> 99%) both purchased from Merck.

All nutrients were weighted and added into glass erlammeyer flasks of 250 mL and 2 L (LINEX) and placed in an incubator (ARALAB, Agitor B 160 E). The compounds were weighted in a Startorius Balance CPA 64 (Startorius Weighing Technology, Germany). Fermentation media were sterilized in autoclave (Uniclave 98 AJE UNI 88). The inoculum was transferred in media at flow laminar chamber (Bisair Instruments - Aura 2000 M.Anc).

At end of the fermentation the cells were centrifuged in falcons of 50 mL (BD, Lda; NUNC VWR, Lda) in a centrifuge 5810 R (Eppendorf just falcons of 50 mL) or in alternative in a centrifuge (Sorvall RC6 for 0.3 L flasks).

To release the enzymes, the cells were sonicated in a sonifier (Bandelim Ultrasonic Corporation, Model 250/450) or in alternative the cells were permeabilized with Lysozyme (22770 U/mg) – (AppliChem), and Deoxycholic Acid (Sodium salt) (Sigma Aldrich). The mixture of this cell suspension was stirred during permeabilization by magnetic agitation (J. P. Selecta, SA, Spain).

For dry cell weight (dcw) were used eppendorf's tubes of 2 mL (Deltalab S.L., Spain) and it was used an oven for cells drying (Binder, Germany).

Optical density (OD) and absorbance values were measured in a spectrophotometer (Hitachi U-2000) with a glass cuvette (1 cm of light path).

The mediators Phenazine Methosulfate (AppliChem Germany) and Sodium 2,6-dichloroindophenolate hydrate (Sigma-Aldrich, Germany) were used for determination of enzymes activities. Ethanol and Acetaldehyde were used as substrates of alcohol and aldehyde dehydrogenases PQQ-enzymes, respectively and they were obtained by: Ethanol absolute anhydrous (99.9% v/v) (Carlo Erba Reagents S.A.S. France) and Acetaldehyde (>99%) (Merck, Belgium).

Potassium dihydrogen phosphate (>99.5%) (Chem-Lab, Belgium) and di-Potassium hydrogen phosphate anhydrous (>99%) (Panreac, Spain) were used to prepare phosphate buffers. To wash the cells were used sodium chloride (>99%) purchased from Fisher Scientific.

The electrochemical measurements occurred in a faraday cage obtained from AUTOLAB and with a potentiostat purchased at CH instruments, Electrochemical Analyzer CHI440B (USA). As referenced electrode was used Silver-Silver Chloride (Ag/AgCl) reference electrode 012167 RE-1B from Bioanalytical Systems, Inc (USA), platinum as a counter electrode purchased at CH instruments (Pt counter electrode CHI 129). For working electrode was used tungsten wire 0.25 mm (99.95% metals basis) and graphite felt (99.95% metals basis), both from Alfa Aesar, (Germany). These electrodes were used in a Biofuel Cell assembly.

Experiments of cyclic voltammetry were made in Tris-HCl buffer and CaCl_2 . Tris-base and HCl (31% v/v) was purchased at Fisher Chemical, UK. The calcium chloride (> 99.5%) was obtained from Merck, Germany.

The immobilization of cells and enzymes in carbon felt was used a stock glutaraldehyde solution (23%) in water purchased to Acros Organics, Belgium.

Potassium permanganate (99%) obtained from Sigma-Aldrich, Germany was evaluated as oxidant 0.04 Mm) in a cathodic chamber. Nafion N-117 membrane, (0.180 mm thick) was purchased from Alfa Aesar, Germany. All solutions were made with purified water from a Milli-Q INT 3 water, Milipure Ibérica S.A., Spain.

The solutions of the cathodic chamber were stirred with magnetic agitation (J. P. Selecta, SA, Spain) and circulated by a peristaltic pump (ISM 795C) (Ismater, USA) through the cathodic chamber.

The voltage measurements were made by a voltmeter (2700 Multimeter / Data acquisition system) purchased at Keithley Instruments, USA.

3.2. METHODS

3.2.1. *GLUCONOBACTER SP.33* CULTIVATION

Gluconobacter sp.33 were inoculated in agar petri dishes according to suppliers information, on agar YPM medium, pH 6.0, (g/100mL): yeast extract (0,5), peptone (0,3), D-Mannitol (2,5) and agar (1,2). The cells were incubated for 24h in an oven at 30 °C.

3.2.2. PQQ-ADH / PQQ-ALDH PRODUCTION BY *GLUCONOBACTER SP.33*

Media used to produce PQQ-enzymes

The PQQ-enzyme (ADH/AIDH) were produced by *Gluconobacter sp.33* specie to growth. Different media with following composition (g/L) were tested, such as: **YPM** (pH 6) (Yeast extract – 5, Peptone – 3, D-Mannitol – 25) [70], **Basal Medium** (BM) (pH 5.5) (Yeast extract – 5, D-Mannitol – 10, Diammonium Hydrogen Phosphate – 1, Magnesium Sulfate - 2) [43] and **GYC** medium (GYC) (pH7,4) (Yeast extract – 10, Peptone – 6, Glucose – 10, Calcium Carbonate – 24) [71]. Additionally, glycerol (GLY) 80% (v/v) was added into YPM and BM media, in same concentration than D-Mannitol. For all media the optic density (OD) values are presented as average of at least 3 readings.

All media were incubated with cells, in 250 mL Erlenmeyer shaking-flask with 50 mL operating volume, at 30 °C and 250 rpm shaking speed per 24 hours.

The results of YPM with GLY proved to be the ideal medium to enzymes production. Therefore, in next experiments to produce of PQQ ADH /AIDH, the cells were incubated in 2 L Erlenmeyer shaking-flask, with 250 mL operating volume, in the same conditions (Figure 9). Still, it was prepared a stock of cultures, in which a culture liquid was saved in sterile cryovials 1 mL, with 15% of glycerol and stored in a freezer at -80 °C.

Fermentation profile of *Gluconobacter sp.33*

To obtain a growth curve of *Gluconobacter sp.33*, samples from medium was collected, every hour, and reading their optic density (OD). The spectrophotometer was calibrated with water and the samples were read a 600 nm. The samples (1 mL of culture medium) were diluted 20 times with water, in order to obtained OD values down 0.6. The growth curve was constructed with OD values, in function to time and it was possible to determine specific growth rate of *Gluconobacter sp.33*.

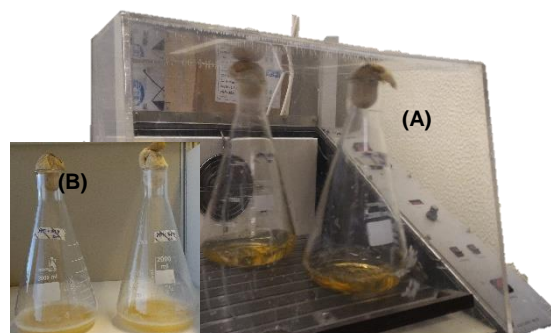


Figure 9: Fermentation of *Gluconobacter sp.33*, at begging (A) and at end of fermentation (B).

Dry cell weight and correlation with cell growth

After fermentation, samples of cells were collected, washed 2 or 3 times and diluted in water and then added into an eppendorf tube of 1.5 mL in triplicate. All tubes were weighted and from the difference of weight in relation to same volume of water in equal eppendorf's tubes before and after drying obtaining the amount of dry cell weight. Drying occurred in an oven, at 60 °C during 72 hours.

From dry cell weight and OD values obtained from several dilutions of stock cell solution, it was possible correlate them in a linear equation, given by $Y = 1.2079 X + 0.0318$, where in x-axis is represented dry cell weight concentration in mg/mL and in y-axis is represented the OD values obtained for several dilutions. Thus, from this correlation was possible to estimate the quantitate of cells produced in each fermentation of YPM + GLY and calculated the specific growth rate of *Gluconobacter sp.33*.

3.2.3. CELL PERMEABILIZATION AND EXTRACTION OF PQQ-ENZYMES (ADH/ALDH)

The cells of *Gluconobacter sp.33* were centrifuged at 5,000 g for 5 min then washed twice with 0.9% NaCl and cell pellet stored at 4 °C in fridge or in alternative frozen at – 20 °C. Then, the pellet of cells was suspended in 0.2 M potassium phosphate buffer (pH 7.0). After that, it was added 10% sodium deoxycholate, for final concentration 0.5%, as well as Lysozyme solution (10 mg Lysozyme, 300 µL 1 M potassium phosphate buffer at pH 7.2, 700 µL of distilled water) for final concentration of 10.5 µL/mL [43]. The suspension was left at 4 °C per 1 hour with slow agitation.

After this, the cell suspension was sonicated for 2 minutes pulsed (5 seconds on, 10 seconds off) at 78 W for cell total rupture [72]. Following this, the suspension was centrifuged again, for 1 hour at 12,000g to separate the supernatant and pellet of cell debris before assay of enzyme activities.

In alternative to the sonication it was also tested cell permeabilization because it was observed that occurred significant loss of enzyme activity during cell rupture. Different final concentrations of sodium deoxycholate (0.5%, 1.0% and 1.5%) were tested in order check the effect of this surfactant on the enzyme activity.

3.2.4. PURIFICATION OF PQQ-ADH /ALDH FROM SUPERNATANT

The cells used to extract PQQ-ADH and PQQ – ALDH enzymes obtained finally in the supernatant after centrifugation were produced in BM medium because it is the medium referenced by other authors [43].

After cells permeabilization process, the supernatant was used for enzyme purification. A 10 % of CaCl₂ solution was added to the supernatant for final concentration of 0.5%, in order to form a calcium phosphate gel, that it was left at 4°C during 45 minutes. Then, the gel was collected by centrifugation for 20 min at 6,000 g and suspended in 0.3 M potassium phosphate buffer (pH 7.2) to initial volume [43].

Finally, to discard the insoluble materials and obtain a supernatant with PQQ-Enzymes was centrifuged again by more 30 min.

3.2.5. DETERMINATION OF ENZYME ACTIVITY OF PQQ-ADH / ALDH

The activity of PQQ-ADH/AIDH was determined by substrate oxidation in spectrophotometric assay method based in the presence of electrons acceptor, 2,6 – Dichlorophenol indophenol (DCPIP) and Phenazine Methosulfate (PMS) with respective substrate, ethanol and acetaldehyde respectively (Figure 10).

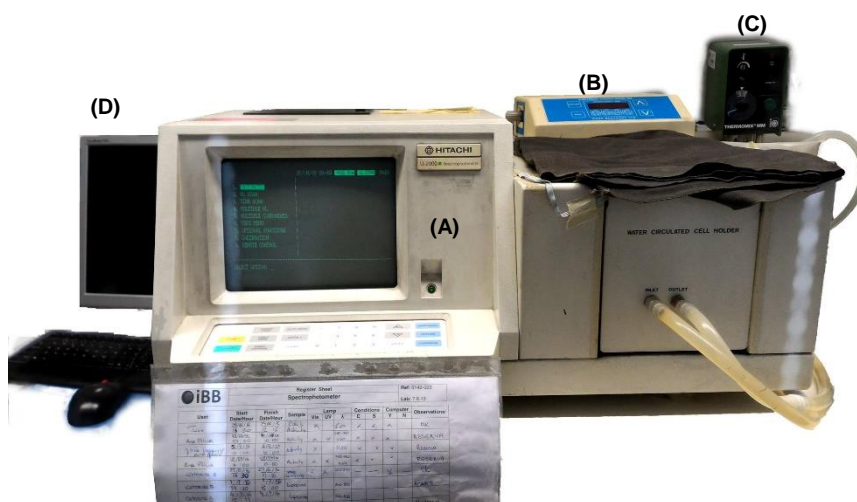


Figure 10: Spectrophotometer used to determination of enzyme activity. (A) Spectrophotometer (B) Agitation control inside of cuvette (C) Temperature control and (D) Data acquisition system.

The activity of enzymes was performed at controlled temperature 37 °C and in 3 mL cuvette with stirring of 70 rpm. The assay method is carried out with 20 µL of diluted sample, potassium phosphate buffer (37.5 mM, pH 7.3), PMS (60 µM), DCPIP (35 µM) and substrate (0.02 M). The absorbance was read at 600 nm during 3 minutes for each sample.

The stability of enzymes at 4°C was carried out by measuring enzyme activity along 6 weeks. The study of stability of enzymes activity was performed with same stock of cells produced with YPM + GLY medium storage at 4°C.

All values of enzymatic activity were calculated according of Beer- Lambert law [73] (equation 11).

$$Abs = \epsilon l [P] \quad (\text{Equation 11})$$

Where, **Abs** is absorbance at 600 nm; ϵ is molar absorptivity of colorimetric compound and **[P]** is concentration of the absorbing species. Through spectrophotometer measurements it is possible to obtain variation of Abs over the time. Thus, divided all equation by time and reorganized the equation to $d[P]/dt$, variation of product concentration over time, i.e, activity of enzyme (ACT), the equation is according by equation 12.

$$\frac{dP}{dt} = \frac{\frac{dAbs}{dt}}{\epsilon l} \quad (\text{Equation 12})$$

In literature is referenced that $\epsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ and as conversion factor, 0.0476 mM/min, for PMS and DCPIP as mediators and OD values read at 600 nm [74]. The length of cuvette is 1 cm, the equation 12 can be write as shown equation 13.

$$\frac{dP}{dt} = \frac{d Abs}{dt} \times 0.0476 \text{ mM/min} \quad (\text{Equation 13})$$

However, as **one enzyme unit (U)** is defined as the amount of enzyme that converts 1 μmol of substrate per minute per mg of protein [75], the values of enzymatic activity were calculated as observed in equation 14:

$$ACT\left(\frac{U}{L}\right) = \frac{dP}{dt} = \frac{d Abs}{dt} \times 47.6 \mu\text{M/min} \quad (\text{Equation 14})$$

The redox reactions that occur involving PQQ-enzymes in the supernatant or whole *Gluconobacter sp.33* cells for measuring of enzymatic activity can be observed bellow in Figure 10.

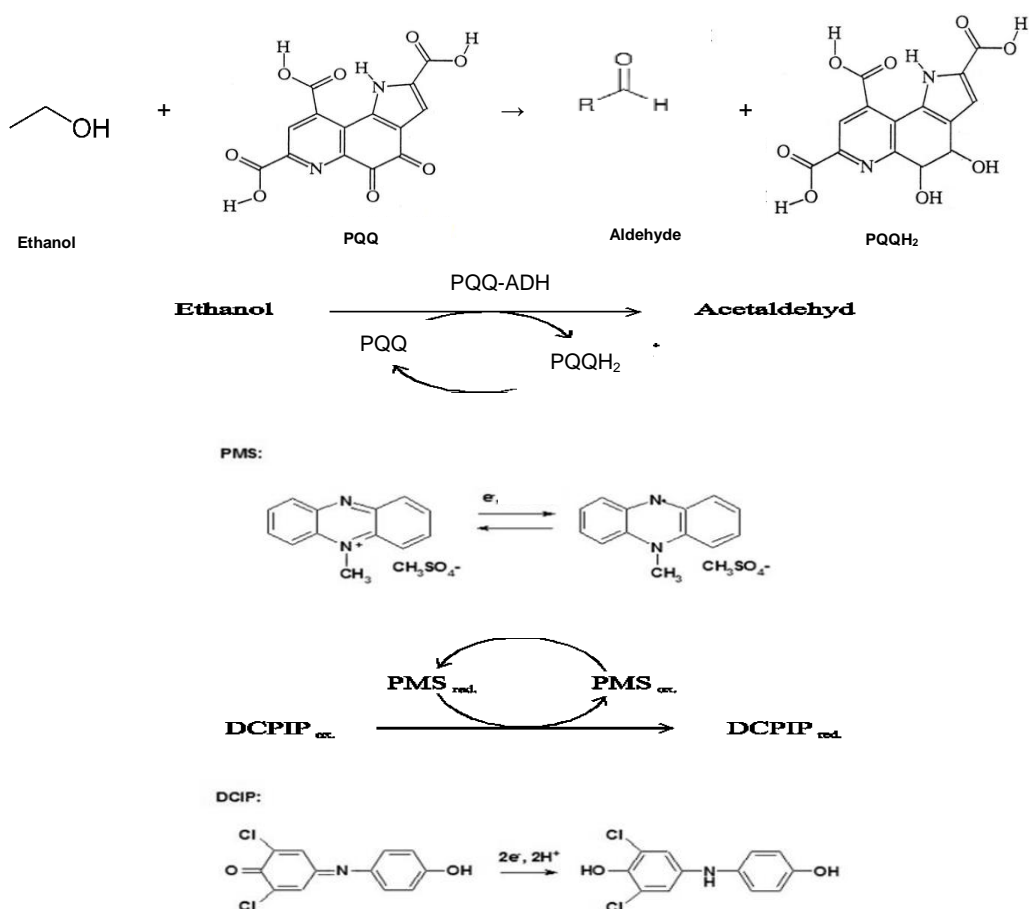


Figure 11: Enzymatic and redox reactions that occur for measuring enzyme activity. The ethanol is oxidized by PQQ-ADH enzyme to acetaldehyde while PQQ (co-factor) is reduced which is regenerated by reduction of PMS transferring the electrons to DCPIP (blue colour) when reduced is characterized by slightly green colour or colourless for total DCPIP consumption. Adapted from [78].

3.2.6. PREPARATION OF ELECTRODE FOR ELECTROCHEMICAL MEASUREMENTS

The working electrode based on carbon or graphite felt was prepared for electrochemical measurements, i. e., cyclic voltammetry and chronoamperometry.

The working electrode consists in a piece of carbon felt with 1 cm width \times 1 cm deep \times 5 cm length, as can be seen in Figure 12. The surface area of electrode is 22 cm². It was left 1 day submerge in water, after removed excess of water and it was left another day with enzyme solution obtained from supernatant or whole *Gluconobacter* cells in a fridge at 4 °C, in order to avoid loss of activity. Then, the electrode was immersed in glutaraldehyde solution, with different concentrations (0.5% and 1.0%), for 1 hour. The results with 0.5% glutaraldehyde showed be ideal condition for obtaining an active and stable working electrode.



Figure 12: Working Electrodes prepared for electrochemical measurements.

The enzyme immobilization using gelatine (the same procedure than Ion Jelly) was also evaluated. 520 μ L of potassium phosphate buffer (50 mM, pH 7.3) and 80 mg of gelatine (added after reaching 60 °C), was stirred for 15 min at 60 °C in a glass flask with a plastic stopper. Then, the solution was cooled to 40 °C and added the enzymatic solution from supernatant. The mixture was stirred at 40° C during more 10 min and transferred to working electrode [65,75].

Finally, all working electrodes were stored in glass flask with potassium phosphate buffer (50 mM, pH 7.3) until it be used at 4 °C. For measurements of cyclic voltammetry and chronoamperometry assays was used the working electrode with immobilized enzymes or cells and respective control after treatment with 0.5 % glutaraldehyde.

3.2.7. INSTRUMENTATION AND ELECTROCHEMICAL MEASUREMENTS

The assays of electrochemical measurements were conducted in a single compartment cell containing 0.2 M Tris-HCl buffer pH 7.5 and 1 mM CaCl₂ in order to observe DET mechanism in *Gluconobacter sp.33* cells and enzymes from supernatant [75]. Also, the assays were performed with KNO₃ 0.1 M and potassium phosphate buffer 50 mM, pH 7.3, but they were not counted because the buffer appear interfere with

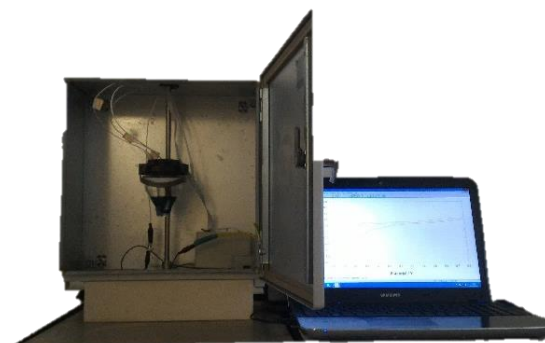


Figure 13: Faraday Chamber – used to electrochemical measurements (cyclic voltammetry and chronoamperometry).

results [77]. Similarly, MET mechanism was tested with *Gluconobacter sp.*33 cells and enzymes from supernatant, using PMS and DCPIP as mediators, in the same concentrations that in determination of enzyme activity, 60 μM and 35 μM , respectively.

Using a silver/silver chloride, as the reference electrode, platinum, as the counter electrode and the tungsten wire inside of carbon felt as working electrode, cyclic voltammetry was performed at scan rate of 5 and 10 mV s^{-1} and range from -0.4 V to 0.8 V in a faraday chamber (Figure 13).

Chronoamperometric experiments were conducted with the consecutive additions of substrates, ethanol from 2 mM to 10 mM and glycerol from 2 mM to 18 mM at fixed oxidative potential of - 0.19 V in relation to SCE.

All cyclic voltammograms shown in this work were adjusted to the reference saturated calomel electrode (SCE), because in literature similar studies with these enzymes used SCE electrode as reference electrode [75]. As redox potential of SCE electrode is + 0.244 V and silver/silver chloride electrode is + 0.197 V, all experimental voltammograms were corrected in -0.047 V, i.e., all voltammograms were moved in -0.047 V.

The peaks of current intensity were calculated by software CHI 440B – Time-Resolved Electrochemical Quartz Crystal Microbalance, version 11.04, CHI instruments (USA). For all current peaks were drawn a tangent line (base line), and obtained the highest current value through another line that indicated the highest current point, in each voltammogram of all experiments of cyclic voltammetry. Bellow, in Figure 14, it is possible to see an example of determination of on peak of current.

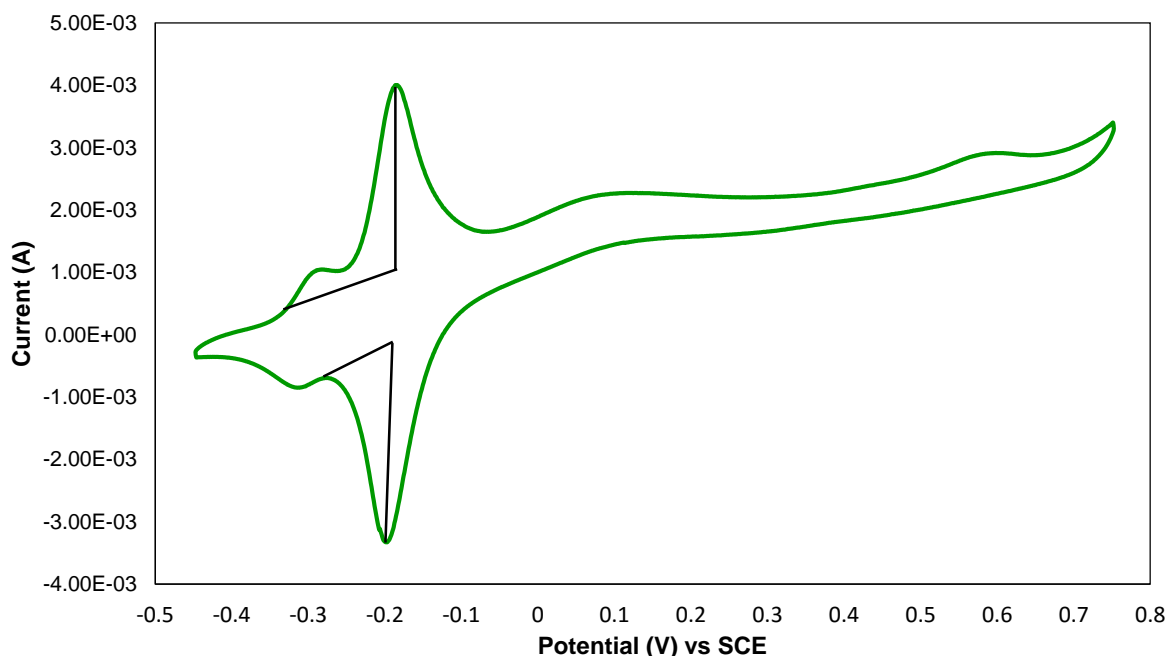


Figure 14: Example of determination of current peaks in voltamograms obtained by DET and MET mechanisms in Cyclic Voltammetry.

For all voltammograms only are presented the 3 and 4 segments of experiments, in order to have a better lecture of results, as illustrated in Figure 15.

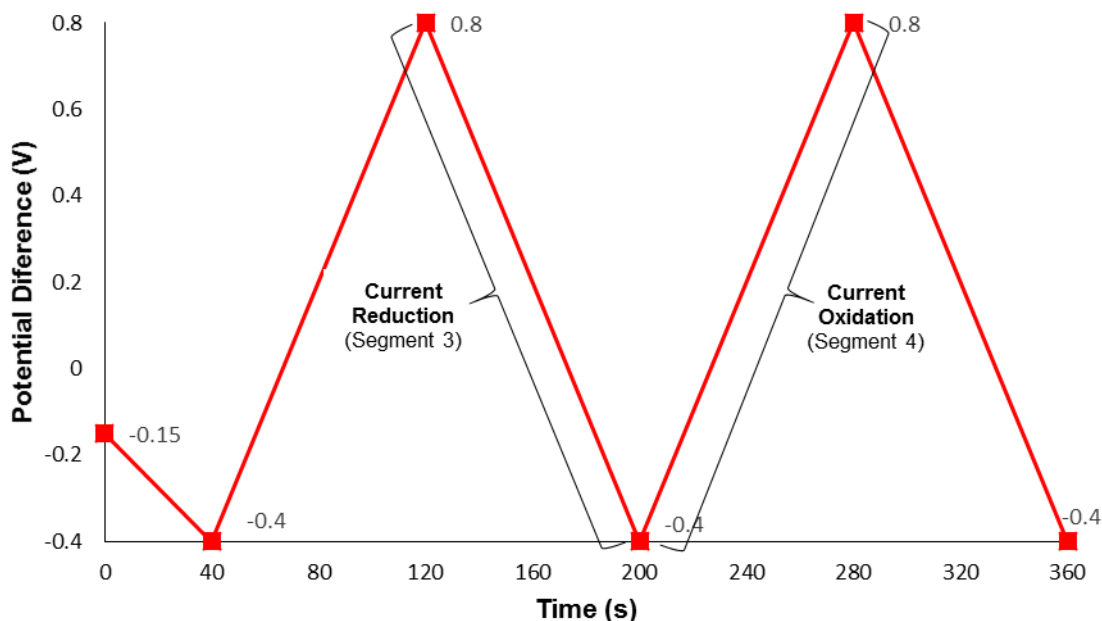


Figure 15: Example of segments represented in all voltammograms in cyclic voltammetry.

3.2.8. STRUCTURE AND ASSEMBLY OF BIOFUEL CELL

BioFuel Cell is composed of several elements mainly an anodic chamber, a cathodic chamber (E) and other secondary constituents needed for their efficient performance, control and monitoring of energy production, as can be observed in Figure 16.

In the reactor that constitute the BioFuel Cell (A) there are one upper anodic chamber (A1) and a bottom small cathodic chamber (A2) each one with an electrode based on carbon felt layer inserted with a tungsten wire to conduct the electrons through the external circuit (Figure 16-G). These two chambers in reactor are separated by a nafion membrane, selective protons membrane, as they need to be isolated and allow the transfer of H^+ generated in the bioanode to the cathode where they react with the electrons and oxidant agent in the cathodic chamber. Thus, there is a structure like carbon felt / nafion / carbon felt (J). During the substrate consumption the reactor was covered by aluminum to avoid oxidation of mediators from nature light in MET mechanism experiments.

The bioanode constituted by carbon felt with immobilized *Gluconobacter* cells is fixed inside of the reactor (F) and for the energy production is then added the electrolyte solution with substrates (DET mechanism) or containing also the mediators (MET mechanism) all mixed slowly with a stirring to improve the mix and diffusion of substrates.

Cathodic chamber (E) is a small compartment in bottom of the reactor which consists basically the cathode of the carbon felt layer in contact with a circulating electrolyte solution (D) of air saturated water or in alternative a potassium permanganate solution (0.04 mM) to improve electrons transference in BioFuel Cell, and check and minimize the oxygen transfer limitations. These electrolyte solutions are pumped via a peristaltic pump (C) and both solutions were agitated in the storage and circulation dark vessel (B).

The electrons collected in the bioanode, i.e., pass from the carbon felt surface layer to a tungsten wire that establish the connection through an external resistance of 1000 Ω with the cathode and close the electric circuit. Data acquisition consisted in measure of voltage difference between the two bioanode and cathode using a voltmeter (H) and the values are registered in a computer (I), every 5 min.

The assays of power production were performed in an electrolyte solution of potassium phosphate buffer 50 mM, pH 7.3 and successive additions of 200 mM of substrate.

In order to stabilize the signal, the experiments were initially left without substrate during 24 h. In MET experiments were added the mediators, in the same concentrations that one used in determination of enzyme activity, PMS 60 μ M and DCPIP 35 μ M, respectively.

The values of current intensity were determined by equation 1. The surface area of bioanode (12.57 cm²) was estimated according available and wet diameter of carbon felt (~ 4 cm) inside of the reactor for determination of power density (equation 4).

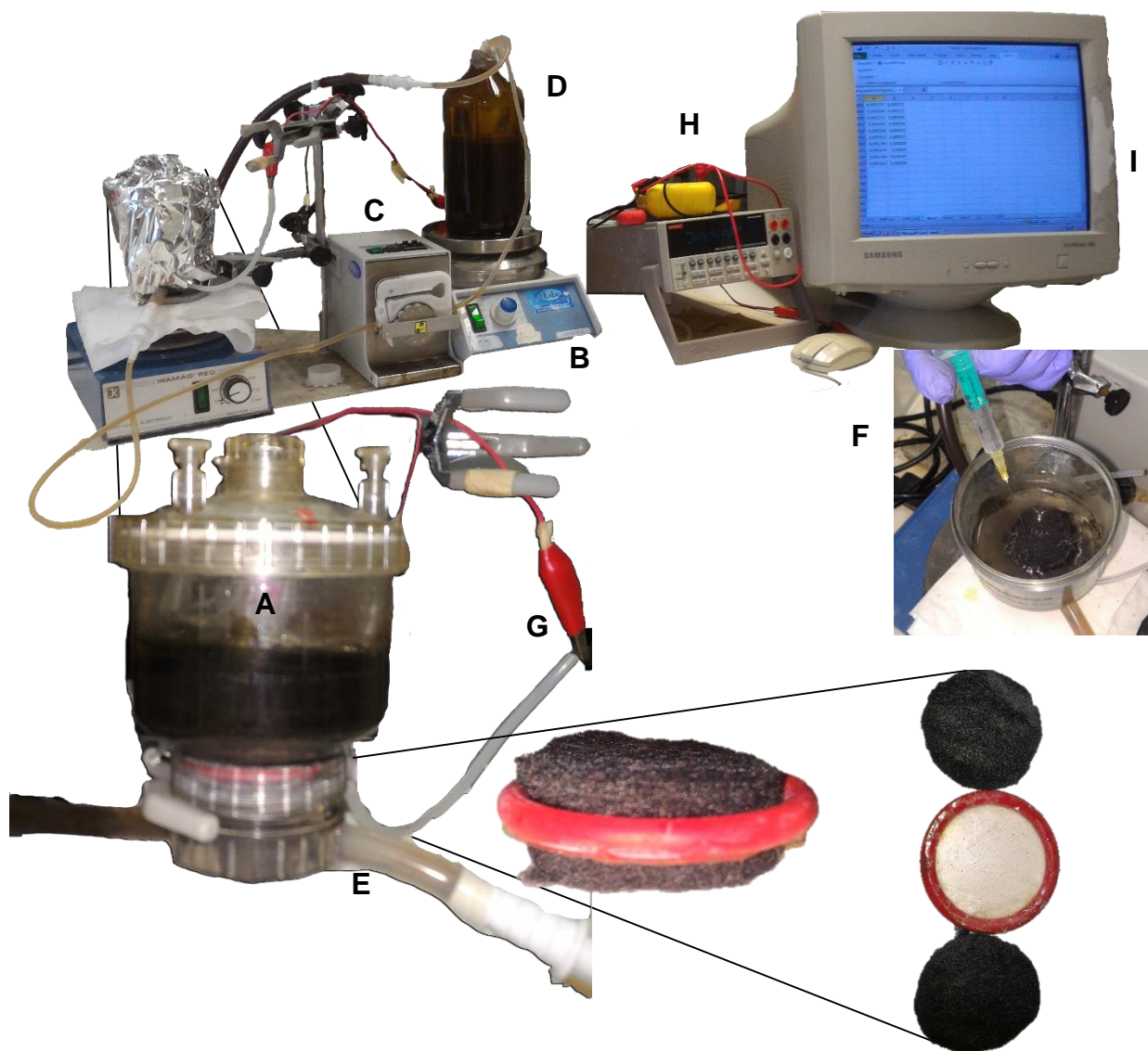


Figure 16: Composition of BioFuel Cell – A: Anode Chamber B: Stir Plates C: Peristaltic Pump D: Storage of circulation liquid in cathode E: Cathode F: Cells Immobilization on carbon felt surface G: Tungsten wires H: Voltmeter I: Data Acquisition System

CHAPTER 4: RESULTS AND DISCUSSION

4.1. PQQ-ADH / ALDH PRODUCTION BY *GLUCONOBACTER SP.33*

4.1.1. MEDIA USED TO PRODUCTION OF PQQ-ADH / ALDH

Gluconobacter sp 33 cells were initially grown in agar petri dishes for 20h at 30 °C and then it was observed their morphology (Figure 17).



The PQQ-ADH and PQQ-ALDH are produced by *Gluconobacter sp.33* and in order to understand which best conditions in terms of bacteria growth, different media were tested, such as: YPM, BM and GYC. YPM and BM medium were also supplemented with glycerol and glycerol with more D-Mannitol. As results of bacteria growth, the values of final OD for all medium it was measured (table 5).

Figure 17: Cells culture of *Gluconobacter sp.33* in YPM medium

Table 5: Culture media tested for *Gluconobacter sp.33* growth and final optic density obtained.

Medium (*)	pH	OD
YPM	6.0	4.8 ± 0.9
BM	5.5	5.4 ± 1.2
GYC	7.4	2.1 ± 0.1
YPM + GLY	6.0	4.8 ± 0.5
BM + GLY	5.5	3.5 ± 0.7
YPM +GLY+ MAN	6.0	3.8 ± 0.4
BM + GLY + MAN	5.5	3.9 ± 0.3

(*) All media were incubated at 30 °C and 250 rpm of agitation during 24 hours.

BM medium showed to be an ideal medium to *Gluconobacter sp.33* growth, obtaining OD value of 5.4, instead of GYC, which has the lowest value of OD, 2.1 (table 5). In relation to GYC, the reason of it may be due to the high concentration of glucose (10 g/L) that produce catabolic repression or some

organics acids with decrease of pH that inhibited the cell growth. BM medium appear to be the best medium for cell growth however, if compared to values of enzymatic activity obtained at end of the fermentation YPM (9.8 U/mL) proved to be better than BM (6.2 U/mL) (Figure 22).

Based in these first results, attempts were made for induce and enhance the enzymes production by supplement YPM and BM medium with GLY, D-Mannitol and both carbon source nutrients.

Through OD values obtained at end of the fermentation is possible to conclude that YPM + GLY was the medium that presented a higher cell grow in comparison with other supplemented media tested. Also, the enzyme activity proved that YPM + GLY was the best medium. In relation to BM + GLY and BM + GLY + MAN both led to lower values of OD and enzymatic activity, than original medium (BM), as well as, YPM + GLY+ MAN.

Although, OD of YPM + GLY medium was only 4.8 but this presented the best result in terms of enzymatic activity and, for this reason, it was the medium selected for the production of PQQ – ADH and PQQ-ALDH. Moreover, in many studies, [41,75, 77], BM is appointed as an ideal medium to production of PQQ-ADH and PQQ-ALDH, but the values of enzymatic activity or OD values aren't always providing or they are in others unit (U/mg).

Based in these first results, attempts were made for induce the enzymes production by *Gluconobacter* sp 33. With this goal, the YPM and BM media were supplemented with GLY, D-Mannitol and both components. The glycerol was already used to other authors to growth of *Gluconobacter* sp.33 and give positive results, instead that occurred when it was used glucose [79, 80]. Also, it can explain the smaller value of OD in GYC medium.

4.1.2. DRY CELL WEIGHT AND CORRELATION WITH CELLS GROWTH

After confirming the ideal medium for enzymes production, the amount of cells produced in fermentation was determined. This was achieved by dry cell weight, which shown that for 1 mL of cells there were 0.015 ± 0.005 g of cells, i.e., 15.0 ± 5.0 mg / mL (table 6).

Table 6 : Dry weight of cells from YPM + GLY medium

	Weight (g)	
	Before Drying	After Drying
Clear Eppendorf's	0.935 ± 0.026	0.934 ± 0.026
Eppendorf's + Water	1.912 ± 0.007	0.912 ± 0.002
Eppendorf's + Water + Cells	1.933 ± 0.008	0.927 ± 0.009
Cells	0.015 ± 0.005	

Comparing the concentration of dry cells weight to OD values obtained at 600 nm with from several dilutions of cells, a linear equation was obtained, but only valid for OD lower than 1, as can be observed in Figure 18. This equation is given by $Y = 1.208 X + 0.032$, where in x-axis is represented dry cell concentration in (mg/mL) and in y-axis is represented the OD values with several dilutions.

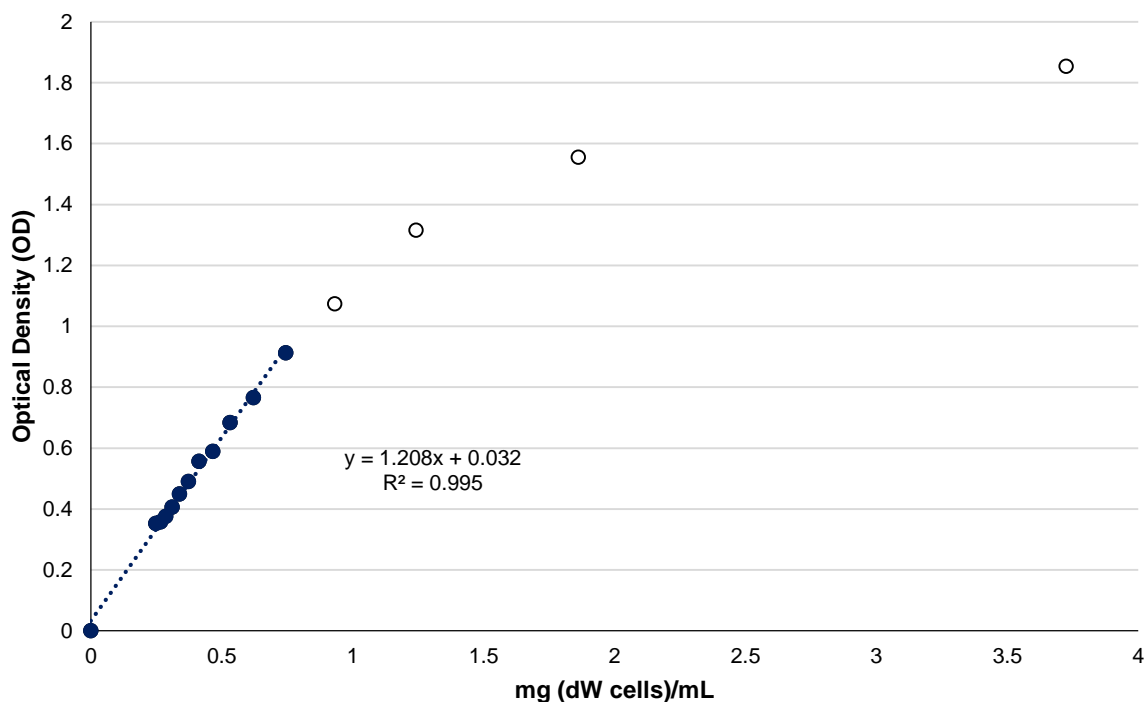


Figure 18: Relation between dry cell weight concentration in (mg/mL) with OD values for several dilutions from original cell suspension.

From this correlation it was possible to determine the specific growth rate as well as it will be possible to estimate the amount of cells per mL, in future fermentations of *Gluconobacter* sp.33 in this medium, (YPM + GLY) or in similar conditions of fermentation.

4.1.3. FERMENTATION PROFILE OF *GLUCONOBACTER SP.33* GROWTH

After selection of ideal medium to produce PQQ-ADH/ALDH, it was verified the *Gluconobacter sp.33*. growth profile (Figure 19). From YPM + GLY medium were performed the fermentation in 2L erlammeyer flask, during 24 hours and with 250 mL of working volume.

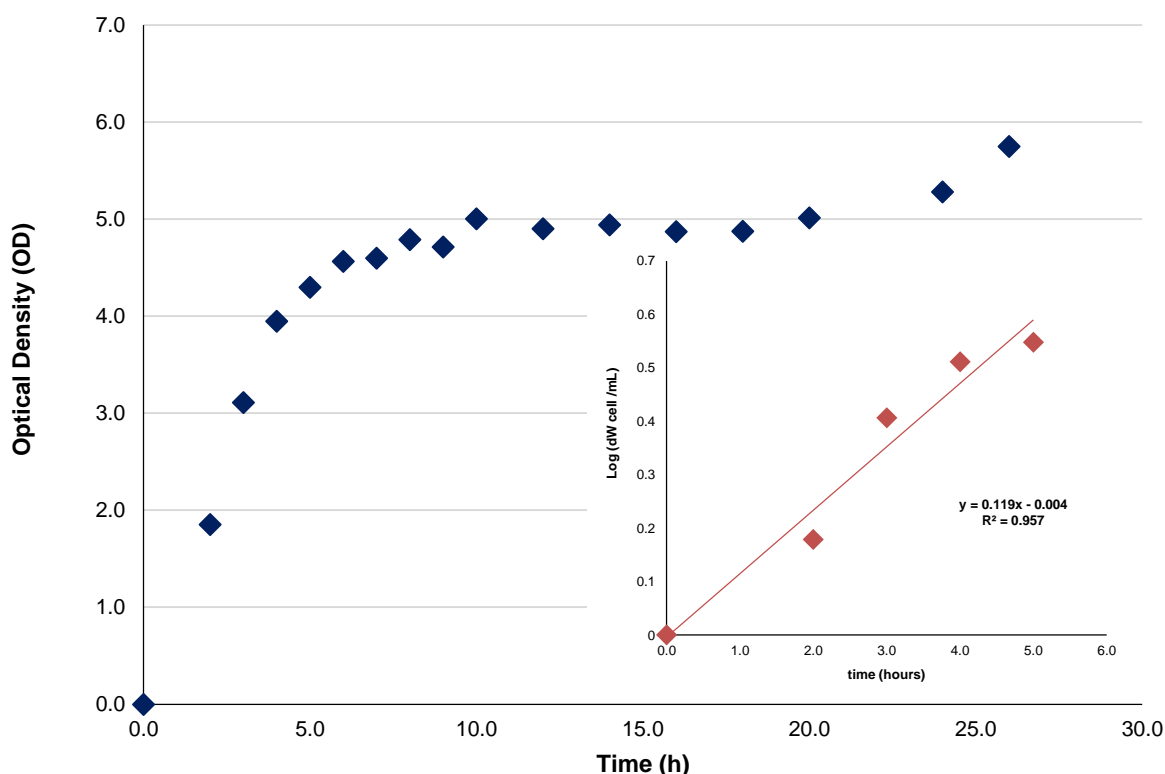


Figure 19: Growth profile of *Gluconobacter sp.33* fermentation, in YPM + GLY medium, for 24 hours at 2 L erlammeyer flask with 250 mL working volume, at 30 °C and 250 rpm.

The exponential growth phase stopped before 10 hours of fermentation and the specific growth rate (μ) was calculated as 0.1187 h^{-1} (Figure 19).

Also, YPM medium was analyzed by other authors, in order to understand which glycerol concentration and pH values were more suitable to *Gluconobacter sp.33* growth [80]. Comparing with our values, in [80] was obtained $\mu = 0.0550 \text{ h}^{-1}$ based in a final OD of 0.345 at pH 5 and $\mu = 0.0556 \text{ h}^{-1}$ based in a final OD of 0.365 at pH 7. Still, these results proved that ideal pH and glycerol concentration for *Gluconobacter sp.33* growth can be 50 g/L and pH 5 or 70 g/L at pH 7 [80]. In this work was used YPM supplemented with 25 g/L glycerol.

In another study the glycerol effect is appointed as a positive factor to bacteria growth but values of OD, μ or concentrations are not providing [79].

4.2. DETERMINATION OF ENZYME ACTIVITY OF PQQ-ADH AND PQQ-ALDH

The measurement of enzymatic activity of PQQ-ADH and PQQ-ALDH in whole cell and supernatant after enzyme extraction was assayed by using a colorimetric method. It consists in reduction of DCPIP (dark blue) by PMS (yellow), which are almost colorless in end of reaction. The reaction starts when it was added the diluted sample of enzyme and in the presence of each specific substrate, i.e., ethanol and acetaldehyde (Figure 20).

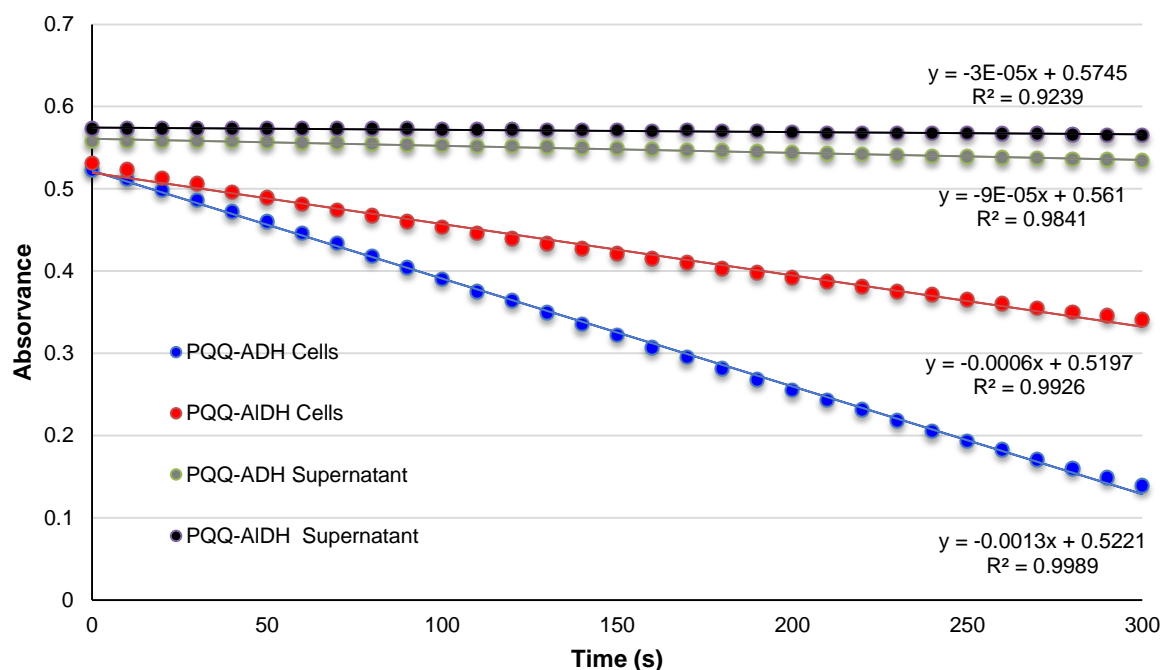


Figure 20: Graphical representation of enzymes reactions in spectrophotometer, through the time.

The velocity of reaction was calculated by linear regression and enzyme activity by Beer-lambert law as referenced in section of methods (Figure 20). One enzyme activity (U) is defined as the amount of enzyme that converts 1 μmol of substrate per minute.

Through this method were possible to determinate enzymatic activity of PQQ-ADH and PQQ-ALDH 8.0 and 11.4 U/mL, respectively. These values are relative to enzymatic activity in the whole cells, while in supernatant the enzymatic activity are lower when compared to cells, i.e., 5.1 and 1.7 U/mL to PQQ-ADH and PQQ-ALDH, respectively.

In referenced articles, values of enzymatic activity are not regularly provided to PQQ-ADH / ALDH [41, 70-72, 80-83]. In some cases, it is presented in U/mg, for example, 35.2 ± 0.5 U/mg and 9.5 ± 0.2 U/mg [75], to PQQ-ADH /ALDH respectively and 3.0 ± 0.9 U/mg and 2.4 ± 0.5 U/mg [77], to PQQ-ADH /ALDH respectively. Although the values are not in same units the method used to determine enzymatic activity was the same than this work [75,77].

However, the enzyme activities of the whole cells are relatively stable with the storage time, as it is possible to see in Figure 21. The enzymatic activity was calculated a long several weeks and verified that after only 2 weeks the enzymes start to loss activity (Figure 21). After six weeks, the values of enzymatic activity of PQQ-ADH / ALDH are 2.3 U/mL and 3.4 U/mL, respectively.

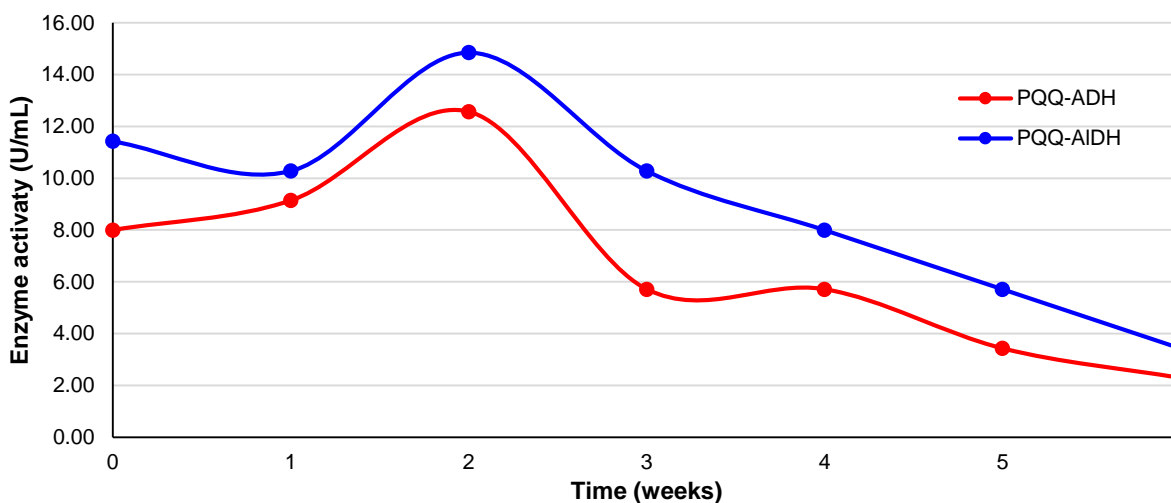


Figure 21: Enzymatic stability of PQQ-ADH and PQQ-ALDH in whole cells. It is represented the enzymes activity over several weeks.

4.3. CELLS PERMEABILIZATION AND PQQ-ENZYMES EXTRACTION

4.3.1. CELLS PERMEABILIZATION PRODUCED IN YPM, BM AND GYC MEDIA

The enzyme extraction, was first tested by simple permeabilization of bacteria cells. During this work, and based in results, some parameters and variables of the process, were optimized to ensure the best enzymatic activity release and accumulated in the supernatant. The treatment of cells permeabilization was performed according reference articles [43,75].

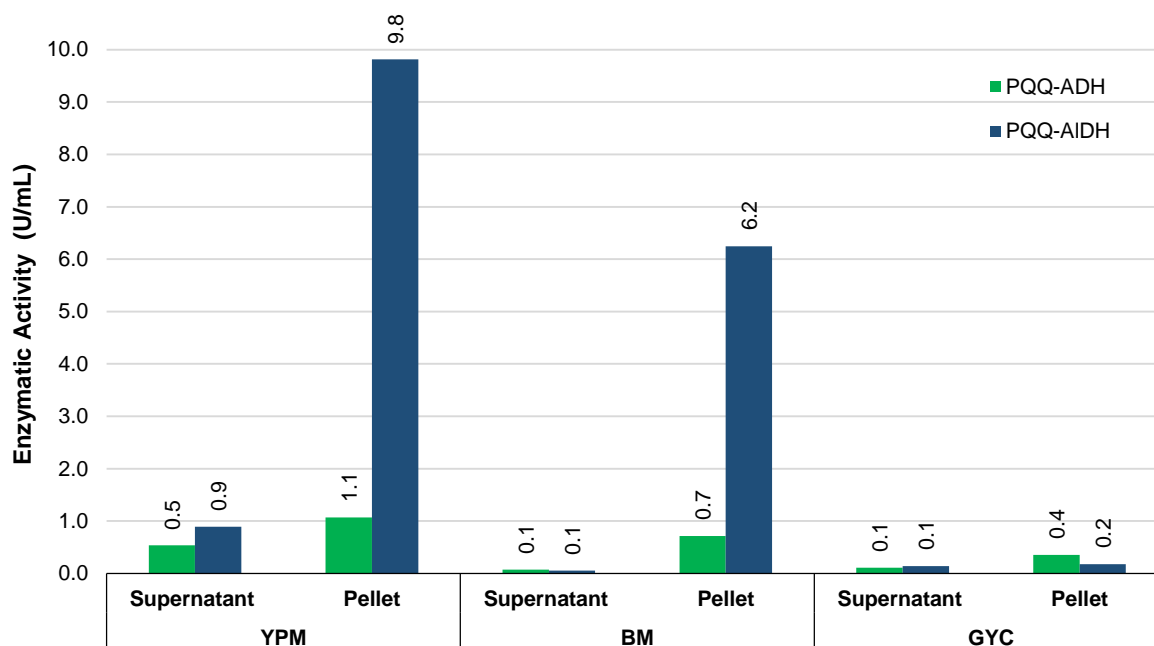


Figure 22: Enzymatic activity in first media used to produce the enzymes, YPM, BM and GYC media. Operation volume 50 mL.

The PQQ-ALDH enzyme activity obtained in the supernatant were much lower than the values of activity remaining in the pellet of cells for all fermentation medium tested (Figure 22) while the PQQ-ADH enzyme activity was negatively affected with the permeabilization method as only a residual value were assayed in the supernatant and pellet of cells. Furthermore, GYC besides not having a satisfactory OD, also didn't show relevant levels of enzymatic activities for PQQ-ADH and PQQ-ALDH enzymes (Figure 22).

As cited before, media like a GYC medium (rich in glucose) can induce inhibition of *Gluconobacter sp.33* growth [79,80-81,83]. Moreover, these results showed the contrary that is suggested by others authors to enzymes production, i.e., BM medium is most referenced medium for PQQ-ADH / ALDH production from *Gluconobacter sp.33* [41,43,75,77], but based on these results of this work this was not observed.

Still, it is possible to conclude that the PQQ-ALDH enzyme activity had remained in cells, i.e., the enzyme extraction wasn't successful, or on the other hand some stage on the cell permeabilization and extraction affected the enzymatic activity. Thereby, the effect of sonication was tested, in order to observe, if with this there were loss of enzymatic activity too.

4.3.2. EFFECT OF SONICATION IN CELLS PERMEABILIZATION

Only in PQQ-ALDH enzyme was verified the sonication effect, because pellet of cells after permeabilization had activity values much higher than PQQ-ADH. The sonication was performed according to [72].

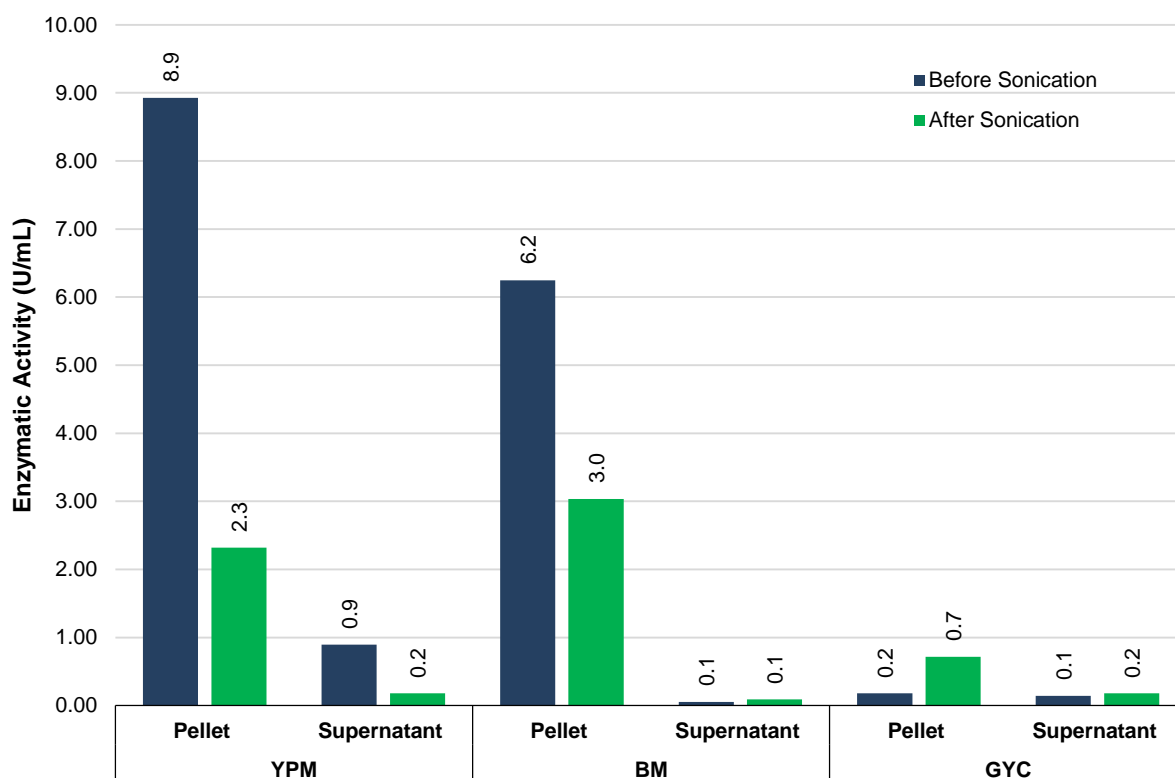


Figure 23: Effect of Sonication in PQQ-ALDH cells permeabilization and extraction. Operation volume 50 mL.

However, by the comparison for YPM and BM media the enzymatic activity decreased 4 and 2 times, respectively, in relation to enzymes activity before sonication (Figure 23). Also, it is important to refer that after cell permeabilization and sonication the enzyme activities continue to be in the pellet of cells and not in supernatant. It is possible to conclude that occurred significant loss of PQQ-ALDH enzyme activity during the sonication step too (Figure 23).

The loss of enzymatic activity can be due to localization of enzymes inside of the cells. As referred before, PQQ-ADH and PQQ-ALDH are located in the cytoplasmic membrane to transfer electrons to ubiquinone Q10 [58-59], as showed in Figure 24. As they are periplasmic enzymes [71] and the permeabilization process (including sonication step) occur in cytoplasmic membrane by membrane breaking, the loss of enzymatic activity can be due to it, leading to denaturation and deterioration of enzyme activities.

Thus, based in these results, the step of sonication was removed of the process of cell permeabilization and enzymes extraction, and so, all enzymes studies henceforward will not have this step, as well as, GYC medium for enzymes production. Then, it was tested the induction in enzymes production from addition of glycerol to media.

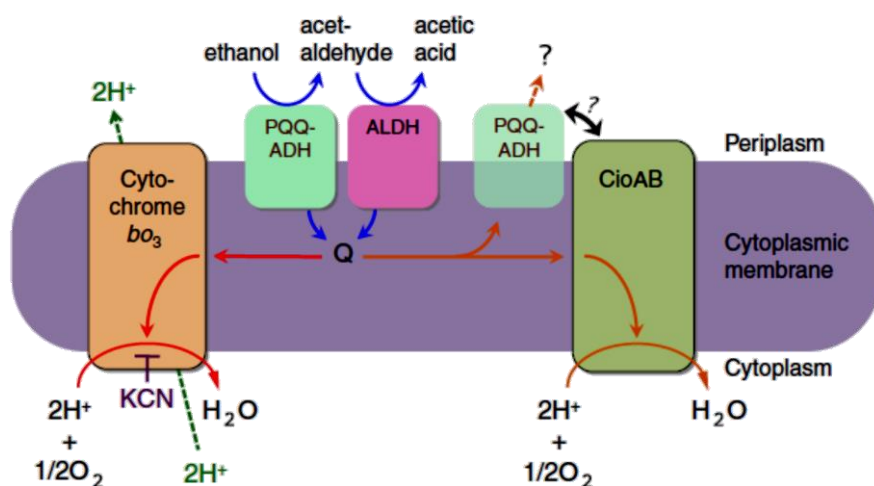


Figure 24: Ethanol oxidation by PQQ-ADH and PQQ-ALDH at the outer surface of cytoplasmic membrane. Adapted from [57].

4.3.3. ENZYMES PRODUCTION BY MEDIA INDUCTION

YPM and BM were selected media for induction the enzyme biosynthesis as they have the highest values of enzyme activity. The induction was tested by adding of glycerol in same concentration than D-Mannitol and double MAN concentration in another fermentation medium, respectively.

In this group of experiments is possible to verify that occurred induction in enzyme biosynthesis, especially in YPM + GLY and BM + GLY + MAN media (Figure 25). The increase of PQQ-ADH enzymatic activity is due to the presence of one alcohol (GLY) and duplicate the concentration of another

(MAN). Even so, in control media, i.e., YPM and BM, the PQQ-ALDH enzymatic activity decreased, when it is compared with enzymatic activity of cells permeabilization in first assay (Figures 22 and 25).

Therefore, it is possible to conclude that PQQ-ADH and PQQ-ALDH enzymes are very sensible during permeabilization treatment as they can deteriorate and resulting in significant loss of enzymatic activity. Or in alternative occurred a lower production of cells in relation to other experiments which could explain the differences of enzymatic activity values specially of PQQ-ADH.

The induced media of BM, proved that there was induction, and instead of YPM, the best result was achieved by supplementation of both alcohols, i. e., BM + GLY + MAN have the best levels of enzymatic activity, although lower than those of YPM + GLY. Still, the control medium, BM, will be used since BM + GLY has higher values of enzymatic activity than BM. This is probably due to amazing value of OD obtained with the BM medium and it is very referenced for several authors as ideal medium to produce these enzymes.

In relation to induced media of YPM, it is clear that YPM + GLY is the best medium, because it has the best result of PQQ-ADH and PQQ-ALDH enzyme activities, not only in YPM induced, as well as, in BM induced media.

YPM and BM were selected media for induction because, they have the highest values of enzyme activity. It was made by adding glycerol in same concentration than D-Mannitol (MAN), and 2 times MAN more glycerol in another medium.

In relation to other BM media, BM + GLY has higher values of enzymatic activity than BM. However, the control medium, BM, will be used for future experiments of enzymes production, on the contrary that these results show because it is very referenced for several authors as ideal medium to produce these enzymes [41,73,75,77] and just for this will be used.

Still, one factor that it is important to refer that *Gluconobacter sp. 33* grew in YPM media supplemented with glycerol, but the induction of enzymes production in these media with *Gluconobacter sp. 33* is not presented [79,80].

Thus, based in the results obtained in this work it is possible to say that occur growth of *Gluconobacter sp. 33* in YPM medium supplemented by glycerol and induction of PQQ-ADH and PQQ-ALDH, because there was an increase of enzymatic activity levels in these media under these conditions, in particularly, in YPM +GLY medium.

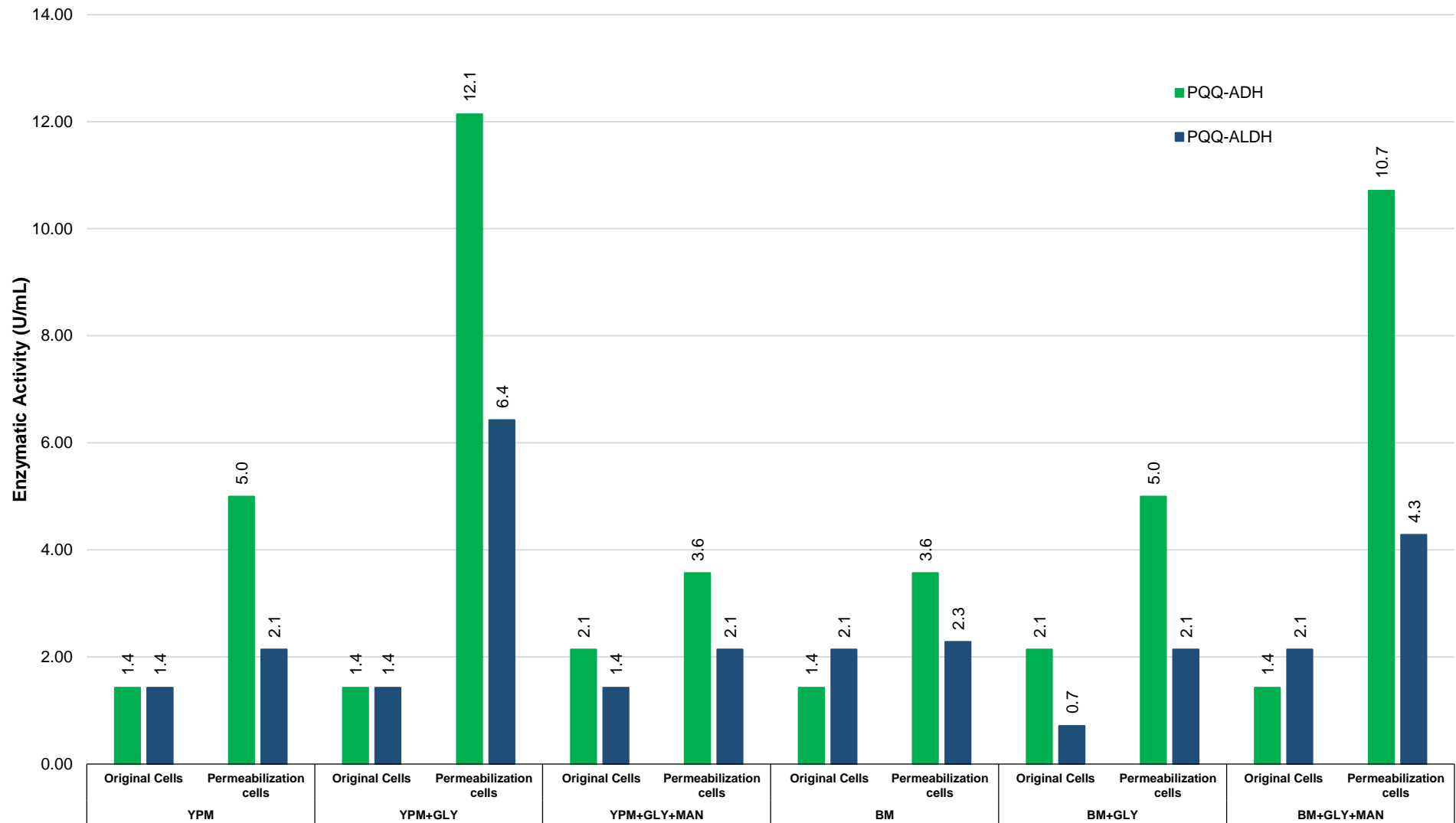


Figure 25: Enzymatic Activity of PQQ-ADH and PQQ-ALDH in original cells in control (YPM and BM) and induced media (YPM +GLY; YPM + GLY + MAN; BM + GLY and BM + GLY + MAN), and after treatment of cellular permeabilization. Operation volume of all experiments based on 50 mL.

4.3.4. PURIFICATION OF PQQ-ENZYMES (ADH/ALDH) FROM SUPERNATANT

Nevertheless, as BM medium is very referenced [41,43,75,77], starting from cells permeabilized it was made the purification of enzymes, i. e., the supernatant purification of BM medium using enzyme precipitation with formation of calcium phosphate gel, in order to understand if there was enzyme concentration and enhance of enzyme activity with this pure enzyme preparation.

The PQQ-ALDH shows to be more fragile than PQQ-ADH as there was loss of this enzyme activity during this process of purification from 2.1 to 0.7 U/mL. Also, PQQ-ADH loses activity over processes, however it doesn't lose as much as PQQ-ALDH, on the contrary, if comparing original cells to purified supernatant, PQQ-ADH increased its enzymatic activity (Figure 26).

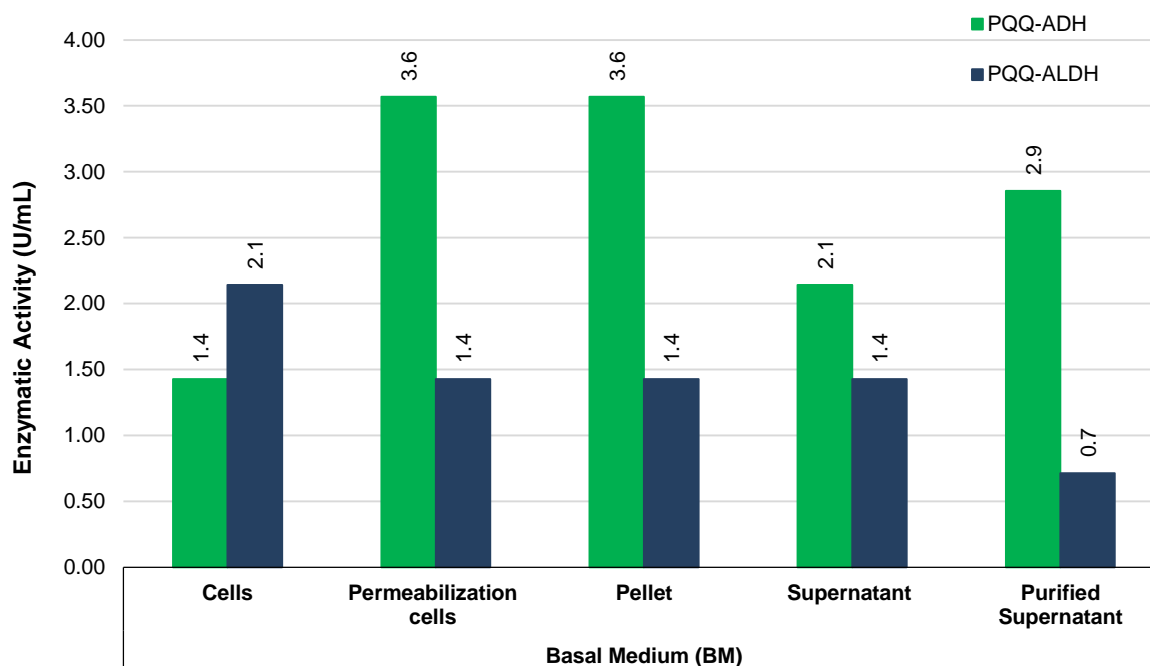


Figure 26: Enzymatic activity in several stages of enzyme purification. Operation volume of all experiments based on 50 mL.

However, the purification process wasn't very efficient, because as it is possible to see in Figure 26, the values of enzymatic activity in purified supernatant are lower than cells permeabilization, and therefore it does not show to be advantage and additionally it is necessary to spend more time and material for purification.

In relation to referenced articles it is not possible to compare the results of enzymatic activity, not even between steps of process because none of these values are provided [41,43,75,77].

4.3.5. EFFECT OF STORAGE IN ENZYMATIC ACTIVITY

The cell storage is normally necessary to minimize loss of enzyme activity before enzyme extraction or purification steps. For this, two different procedures of cells storage were tested, i.e., the cell permeabilization has been made from cells stored at 4 °C in fridge and in alternative frozen at - 20 °C.

According both cell storage procedures and after cell permeabilization, were obtained in pellet of cells and supernatant with higher activity showed for frozen cells at - 20°C independent of the fermentation medium used, i.e., YPM, as well as YPM+GLY and BM + GLY + MAN (Figures 27 and 28). Only BM medium proved to have more enzymatic activity in cells permeabilized from fridge. Additionally, all values of enzymatic activity are higher in supernatant than pellet of cells.

Thus, it is possible to conclude that the best way to store cells before enzyme extraction is in a freezer kept – 20 °C. This conclusion is conforming as it was observed in referenced articles, i.e., cells should be stored at freezer at – 20 °C [41,75,77]. Nevertheless, this step didn't need to be used, in this work, because original and fresh cells were used to incorporate in the bioanode of the Biofuel cells.

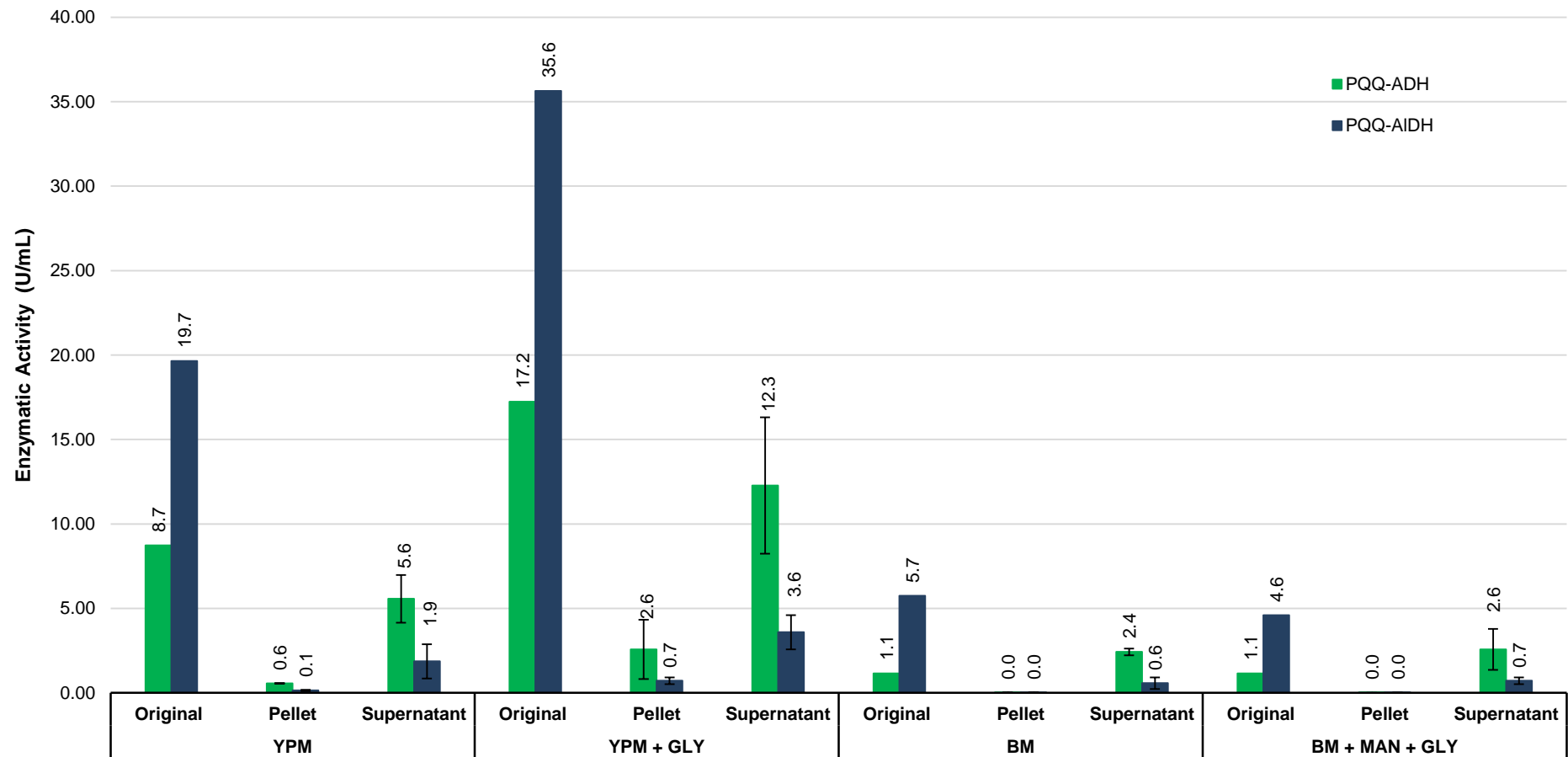


Figure 27: Enzymatic activity of cells stored in fridge at 4°C. It's presented the enzymatic activity in original cells and after treatment of cellular permeabilization, in pellet and supernatant. Operation volume of all experiments based on 250 mL.

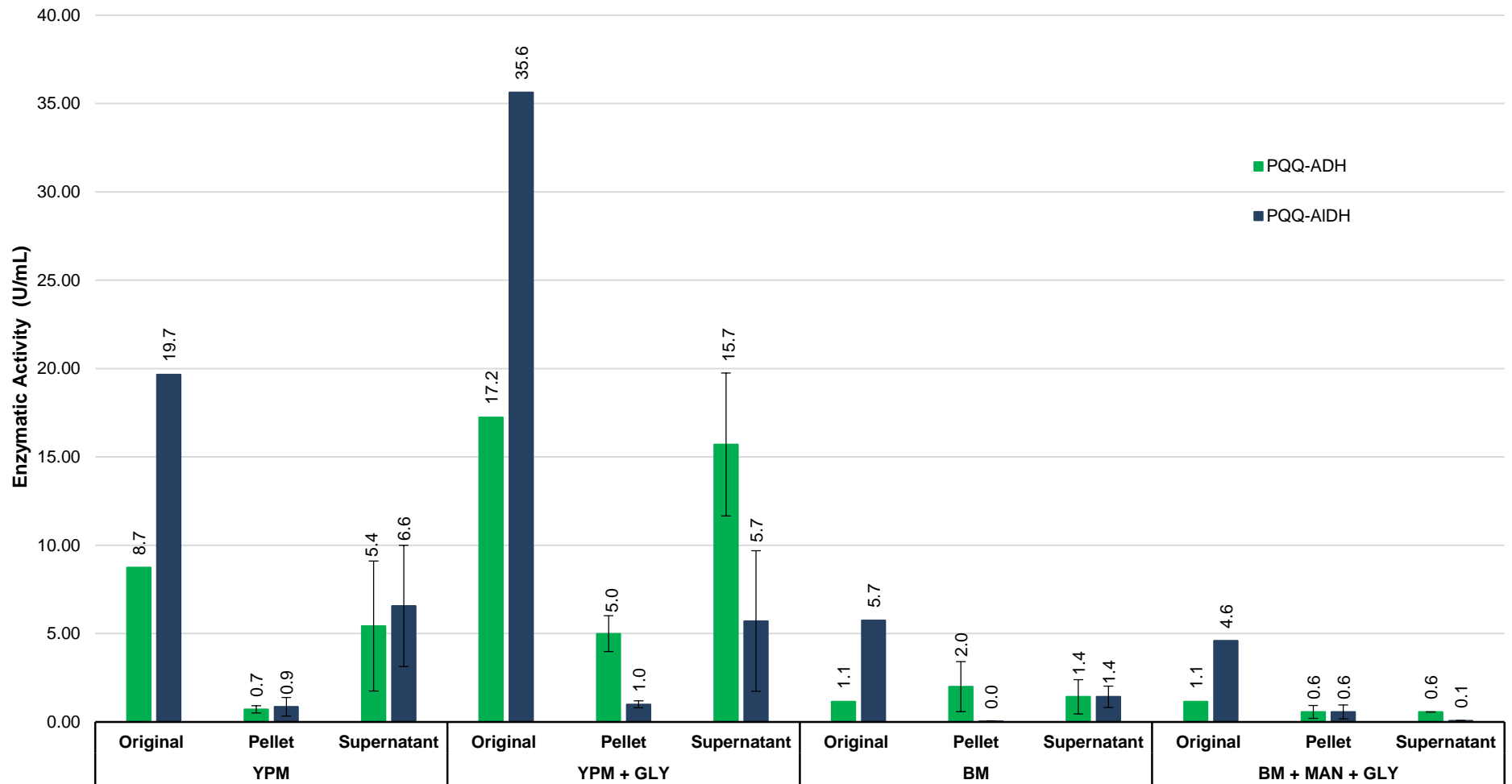


Figure 28: Enzymatic activity of cells stored in freezer at -20°C. It's presented the enzymatic activity in original cells and after treatment of cellular permeabilization, in pellet and supernatant. Operation volume of all experiments based on 250 mL.

4.3.6. EFFECT OF PROPORTION IN CELLS PERMEABILIZATION

After all these studies, the YPM + GLY proved be the best fermentation medium to produce PQQ-enzymes, instated that were pointed to reference articles [43,75,77], so for future work, only this medium was used. The cell produced in YPM + GLY were tested in several percentages of sodium deoxycholate in relation to original cell permeabilization method in order to minimize enzymatic activity loss during this process and, mainly, obtain more active PQQ-enzymes in supernatant.

In Figure 29 shows that original cells have more enzymatic activity than cells experienced permeabilization treatment for all % of sodium deoxycholate used and there is higher enzyme activity in supernatant than pellet of cells except for 1.5% of deoxycholate.

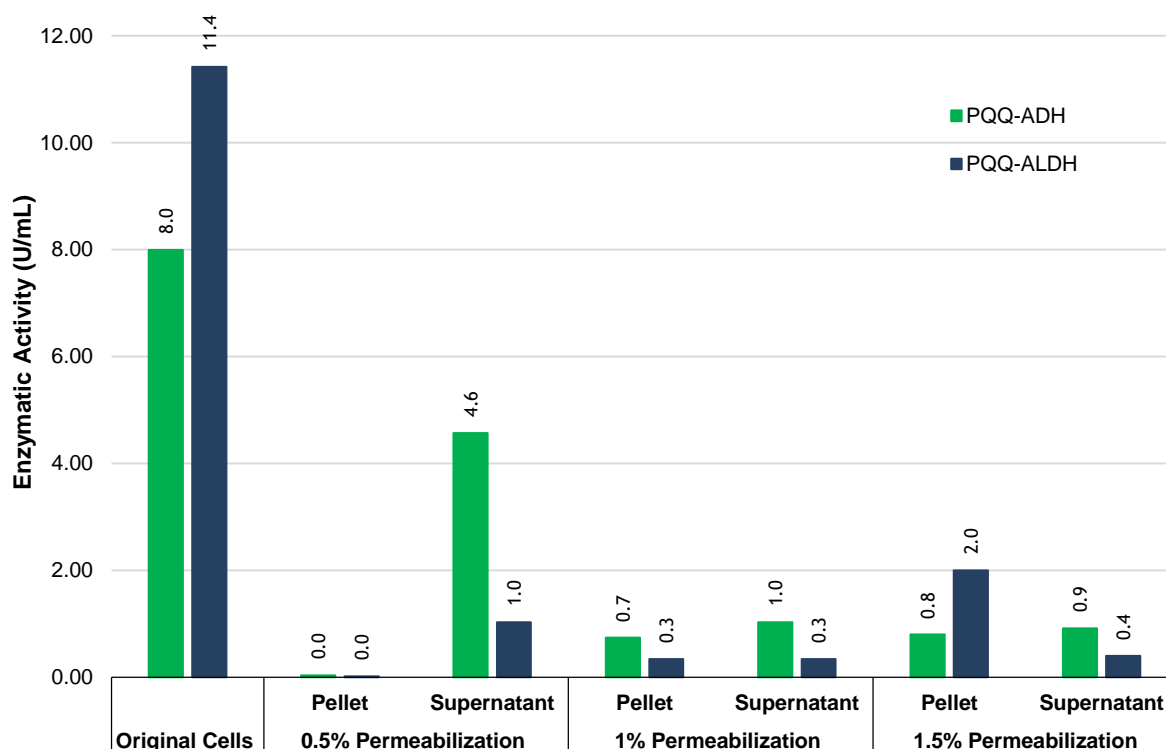


Figure 29: Enzymatic activity of cells from YPM+GLY medium. It's presented in original cells and after treatment of cell permeabilization, with different concentrations, in pellet and supernatant. Operation volume of all experiments based on 250 mL.

Thus, it is possible to conclude that the best concentration that led to better results of enzymatic activity is 0.5% of deoxycholate for this cell permeabilization methodology, as cited by authors [43,75]. Similarly, that was referred previously, it is not possible to compare the values of enzymatic activity.

Furthermore, and according these results with significant loss of enzyme activities was decided to test the Biofuel Cells experiments with the whole *Gluconobacter sp.* 33 cells instead to PQQ-enzymes obtained in supernatant.

4.4. INSTRUMENTATION AND ELECTROCHEMICAL MEASUREMENTS

4.4.1. WORKING ELECTRODE

The working electrodes used for cyclic voltammetry and chronoamperometry were constructed as describe in 3.2.6 *Electrode preparation for electrochemical measurements in chapter 3: Materials and Methods*.

Initially, the working electrode was checked, in order to understand enzyme activity in the whole cell incorporated in the carbon felt. This was tested in a flask, containing the mediators (35 μ M DCPIP and 60 μ M PMS) in same concentration that one used for the determination of enzyme activity, in phosphate buffer 50 mM pH 7.3 and 20 mM of ethanol. The immobilization of whole cells in the carbon felt was tested 0.5% and 1% of glutaraldehyde, and gelatin. For each specific working electrode tested 2 electrodes, one with immobilized cells and other as a control, cell-free.

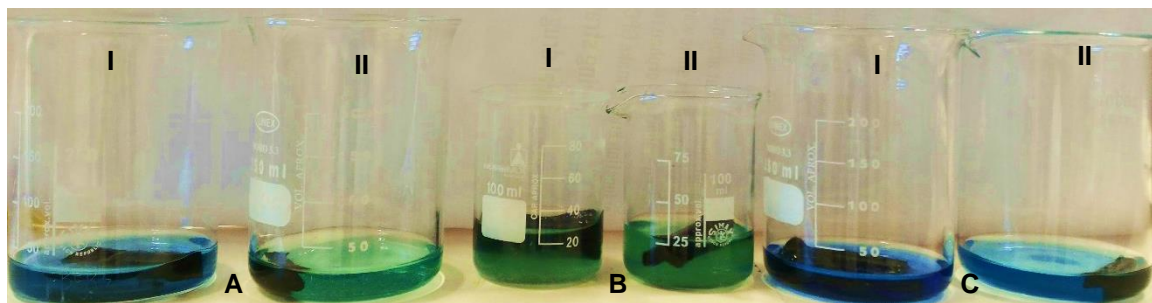


Figure 30: Tests of Enzyme Immobilization: A (0.5 % of Glutaraldehyde): I – Control, II – Cells; B (Gelatine): I – Control, II – Cells; C (1 % of Glutaraldehyde): I – Control, II – Cells;

According the different methods used for cell immobilization it is clear that the cells immobilized in 0.5% of glutaraldehyde show more activity and stability than with 1% of glutaraldehyde as the reaction occurred and the mediators were consumed by changing the initial color from dark blue to slightly blue or green (Figure 30). The immobilization method based on gelatin show a color change developed in both flasks (green color) mainly due to presence of gelatin in both working electrodes and for this method was not consider for future experiments.

Thus, the immobilization method selected for whole cell and enzyme in supernatant in carbon felt was based in 0.5% of glutaraldehyde. First, the whole cells / enzymes are in contacted with the electrode (adsorption), approximately 1 day, and then they are reticulated through a glutaraldehyde 0.5 % solution in carbon felt during 1 hour. Finally, the electrodes are stored in a flask with phosphate buffer 50 mM pH 7.3 at 4 °C until will be used.

4.4.2. CYCLIC VOLTAMMETRY

The cyclic voltammetry was performed to observe and confirm the redox reaction that can occur through the PQQ-enzyme activity produced by *Gluconobacter sp.*33 cells and according two electrons transfer mechanism: Direct electron transference (DET) and Mediator electron transference (MET). In these experiments two different substrates were used - ethanol and glycerol.

In these experiments of cyclic voltammetry, the working electrodes tested were based on immobilization of **cells** (original cells) and **supernatant** (enzymes from supernatant) incorporated in the carbon felt and **free cells** in solution (carbon felt without cells) as working electrode used as control.

DET (Direct Electron Transference) with ethanol

For DET mechanism the working electrodes based on **cells** (Figure 31), **supernatant** (Figure 32) and **free cells** (Figure 33) were tested.

The working electrode with **cells** didn't show a good performance because the current increase is really small in direct electron transference, as shown in Figure 31. However, there is a shift of the peak from 0.05V to 0.17 V for control working electrode and working electrode with cells, respectively for ethanol substrate in the reaction media. Furthermore, it is visible a second characteristic peak of current at ~ 0.6 V. In first peak at 0.17 V, the oxidation current increases with ethanol concentration of 3.30×10^{-4} A, 4.67×10^{-4} A and 4.87×10^{-4} A for **cells**, **cells with 5 mM** ethanol and **cells with 10 mM** ethanol, respectively.

There are a second peak at 0.64 V but it is not very well known its origin. Additionally, there are inversion of current, i.e., this decreases with increase of ethanol concentration, as can be observed by intensity current values, 1.89×10^{-4} A, 1.54×10^{-4} A and 7.66×10^{-5} A for **cells**, **cells with 5 mM** ethanol and **cells with 10 mM** ethanol, respectively.

As in working electrode with **cells**, the enzyme immobilization in carbon felt (**supernatant**) doesn't show also the desired performance and there is small shift in peaks of working electrodes with enzymes in relation to respective control as it can be seen in Figure 32.

Furthermore, also here the working electrode with enzymes (**supernatant**) there were two peaks of current, at ~ 0.1 V and ~0.6 V. In first peak, 0.1 V, the current is bigger in **supernatant 5 mM** ethanol than **supernatant**, however for **supernatant 10 mM** ethanol is smaller one. It can be verified with oxidation current values, 7.57×10^{-4} A, 8.04×10^{-4} A and 4.69×10^{-4} A for **supernatant**, **supernatant 5 mM** ethanol and **supernatant 10 mM** ethanol, respectively.

Also in this case, it presented a second peak at ~ 0.6 V but it is not very well known the origin. However, it is not possible correlate with current and ethanol added, because oxidation current values aren't coherent, 2.34×10^{-4} A, 8.55×10^{-4} A and 1.64×10^{-4} A for **supernatant**, **supernatant 5 mM** ethanol and **supernatant 10 mM** ethanol, respectively.

Finally, the DET mechanism was verified in a solution with **free cells**, as can be seen in Figure 33. The free cells in the electrolyte didn't show any peak of current, i.e., cells in solution did not show direct electrons transfer as expected in comparison with two previous working electrodes with biocatalysts immobilized in carbon felt.

DET (Direct Electron Transference) with glycerol

Lastly, it was tested the working electrodes with immobilized cells (**cells**) on the oxidation of glycerol (Figure 34). The working electrode with **cells** didn't provide a better performance in relation to the similar experiment but with ethanol. Similar to what occurred in cells with ethanol, in cells with glycerol there are 2 peaks of current at ~0.1 V and ~0.6 V. In first peak, ~0.1 V the oxidation current increase with glycerol 5mM but not from 5 mM to 10 mM in opposition with it was observed with working electrode with cells for ethanol. However, the values of oxidation current were very similar and probably in the experimental error assay, i.e., 2.62×10^{-4} A, 2.68×10^{-4} A and 2.68×10^{-4} A for **cells**, **cells 5 mM** glycerol and **cells 10 mM** glycerol, respectively.

Also in this case, it presented a second peak at ~ 0.6 V but it is not very well known the origin. However, the oxidation current decreases with glycerol concentration 7.05×10^{-5} A, 6.71×10^{-5} A and 5.57×10^{-5} A for **cells**, **cells 5 mM** glycerol and **cells 10 mM** glycerol, respectively as it was already observed for ethanol.

Other authors, performed cyclic voltammetry by DET mechanism with PQQ-ADH and PQQ-ALDH purified from a supernatant, and both enzymes produced from *Gluconobacter sp.*33 [75]. The working electrode was made by 3 different types of enzymes immobilization: tetra-butyl ammonium bromide (TBAB) - modified Nafion; octyl-modified liner polyethyleneimine (C₈-LPEI) and cellulose.

All electrodes provide good results in DET. It is clear two peaks, corresponding to oxidation and reduction of enzymes, - 0.05 and - 0.25 V respectively, where it is possible to see a current increase as ethanol concentration was added. The working electrodes presented higher values of current density such as, 90, 80 and 35 $\mu\text{A}/\text{cm}^2$, for TBAB- modified Nafion electrode, C₈-LPEI electrode and cellulose electrode, respectively [75].

Based in this values, it is possible to conclude that DET mechanisms didn't have a good performance in our experiments, the values of current intensity obtained are residual if comparing to reference article. Then, it will be showed the same experiments for MET.

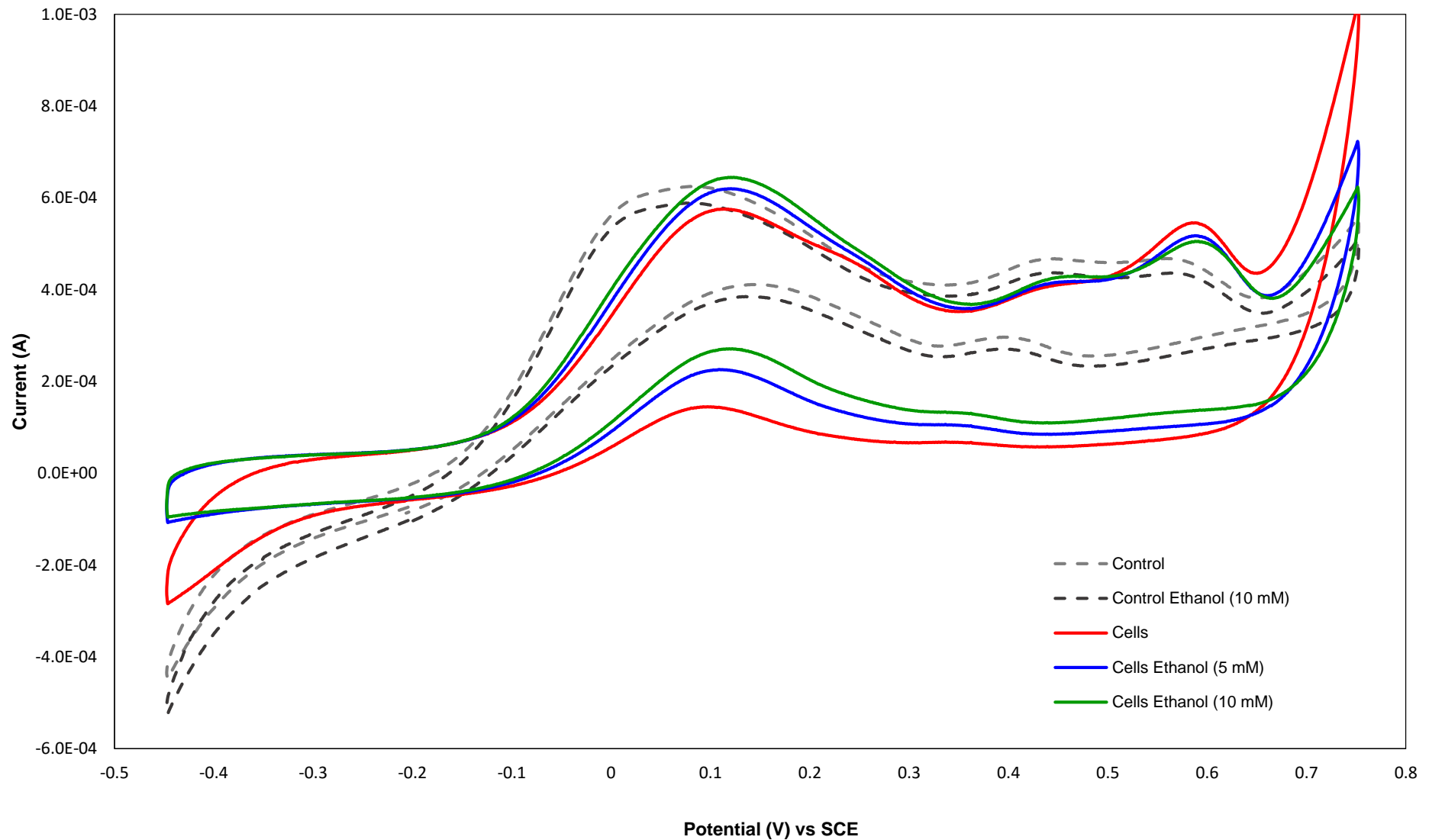


Figure 31: Representative cyclic voltammograms with working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from original cells, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface. Also, it is presented the control of working electrode, i.e., cells free. The assays were made based of DET mechanism, in Tris-HCl buffer and CaCl_2 , with 5 / 10 mM ethanol, pH 7.5 at voltage rate of $0.010 \text{ V} \cdot \text{s}^{-1}$.

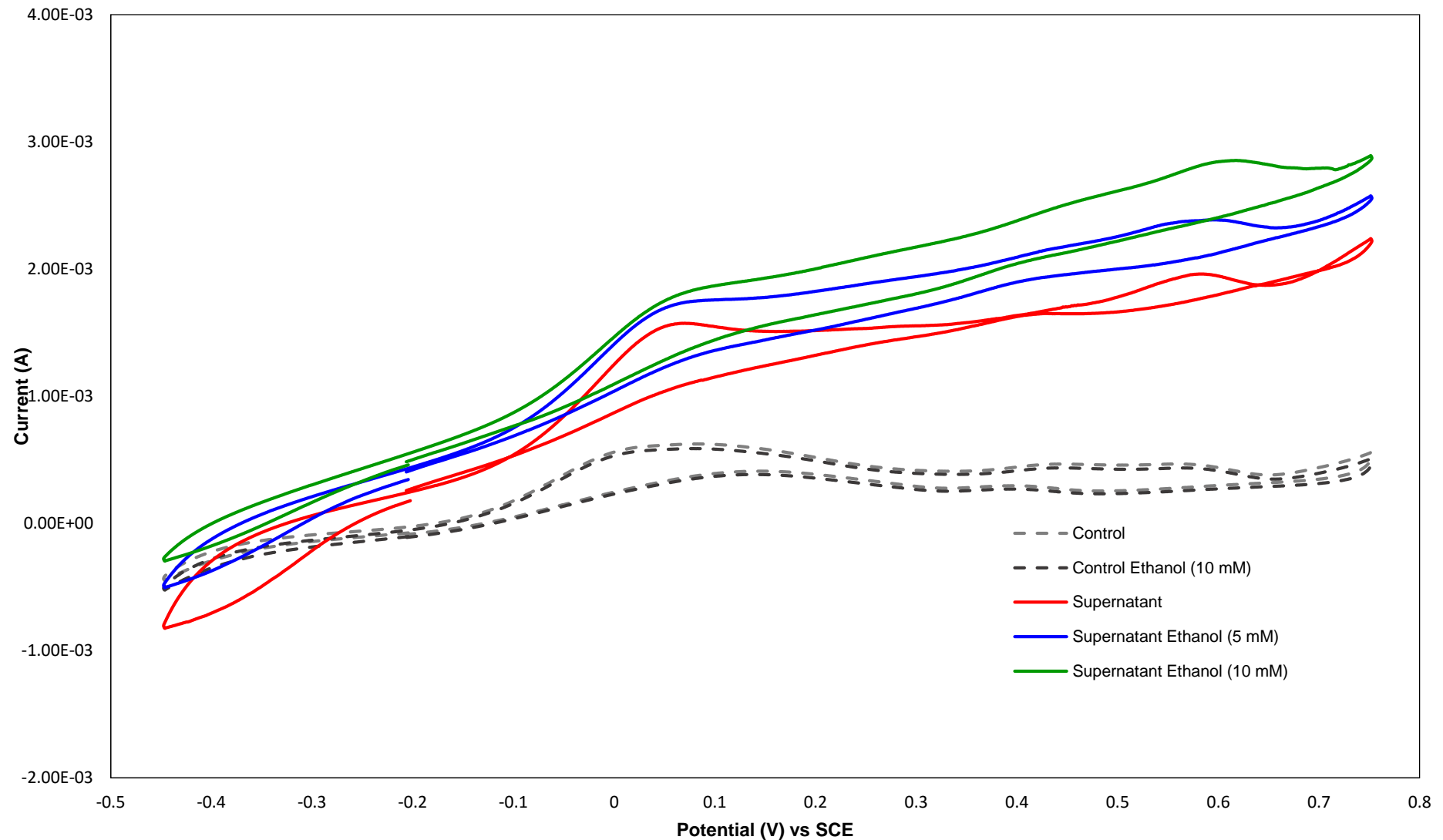


Figure 32: Representative cyclic voltammograms with working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from supernatant, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface. Also, it is presented the control of working electrode, i.e., without cells. The assays were made based of DET mechanism, in Tris-HCl buffer and CaCl_2 , with 5 / 10 mM ethanol, pH 7.5 at voltage rate of $0.010 \text{ V} \cdot \text{s}^{-1}$.

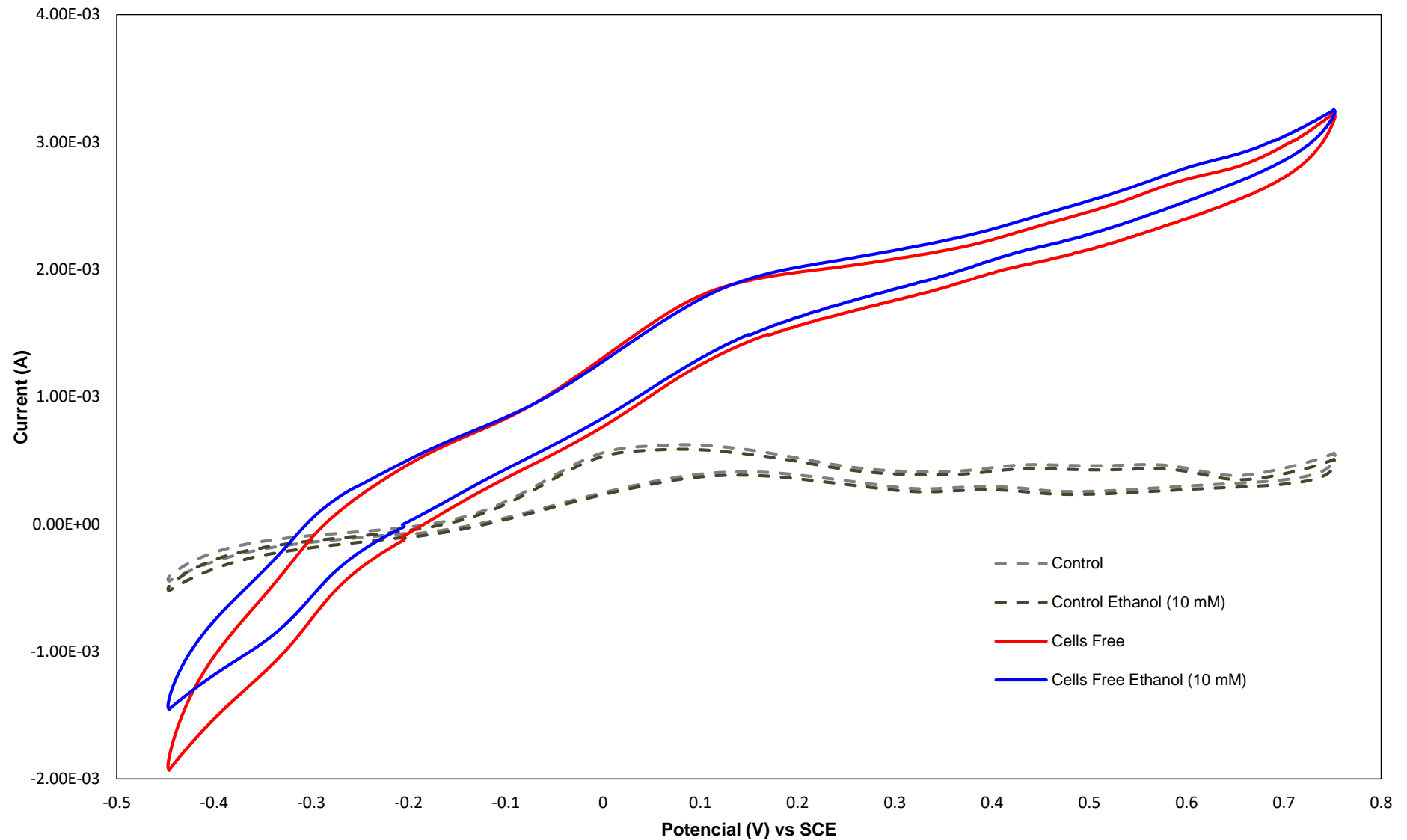


Figure 33: Representative cyclic voltammograms with working electrodes without biocatalysts (cells or enzymes) but tested for absence of cells (control) versus the presence of free cell in solution of the electrolyte (Cells Free). The assays were made based of DET mechanism, in Tris-HCl buffer and CaCl_2 , with 5 / 10 mM ethanol, pH 7.5 at voltage rate of $0.010 \text{ V} \cdot \text{s}^{-1}$.

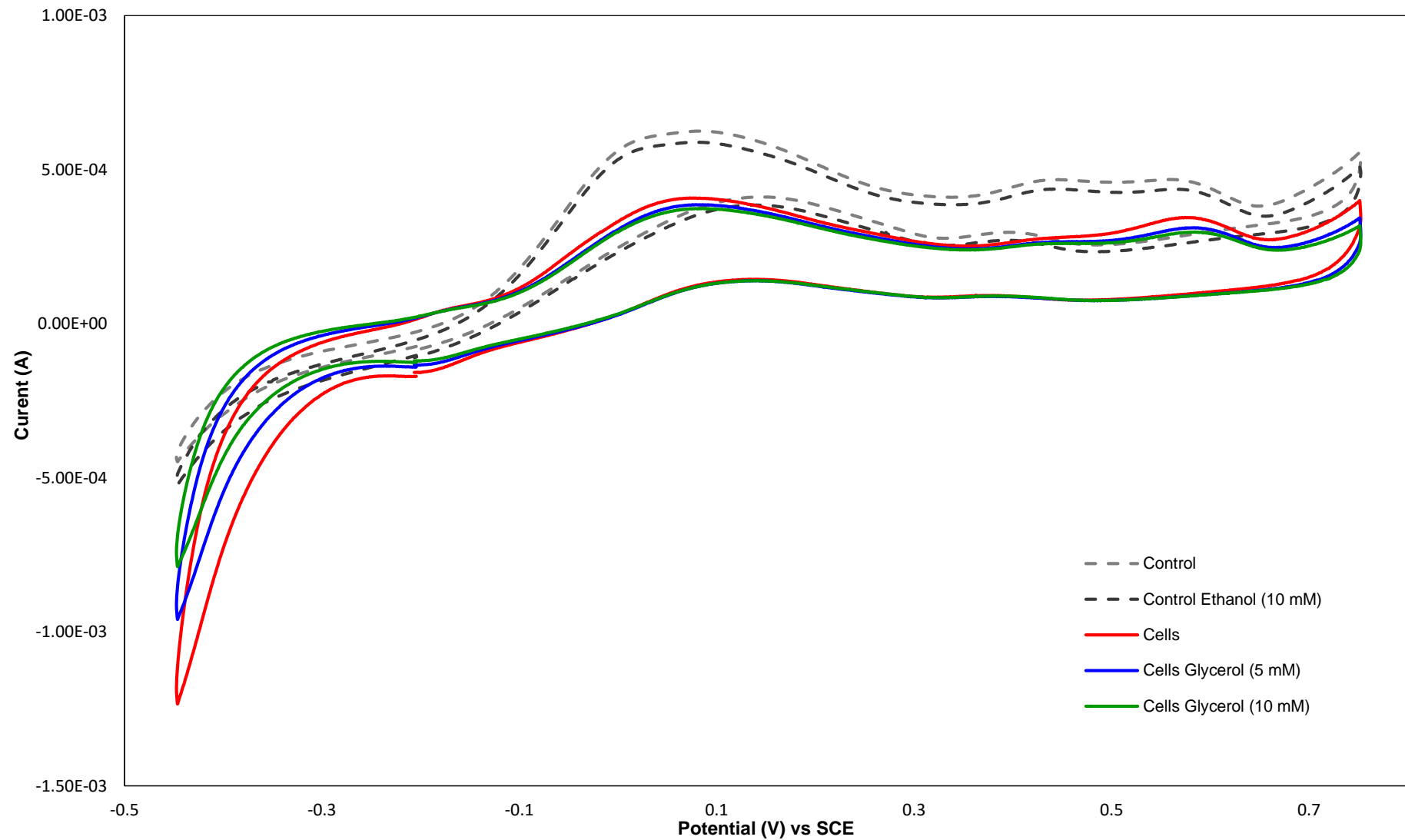


Figure 34: Representative cyclic voltammograms with working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from original cells, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface. Also, it is presented the control of working electrode, i.e., cells free. The assays were made based of DET mechanism, in Tris-HCl buffer and CaCl_2 , with 5 / 10 mM of glycerol, pH 7.5 at voltage rate of $0.010 \text{ V} \cdot \text{s}^{-1}$.

MET (Mediator Electron Transference) with ethanol

For MET mechanism was tested for PQQ-enzymes using whole *Gluconobacter* cells and enzymes present in the supernatant. In Figure 35, it is possible to observe the different colors in the electrolyte solution before and after perform the cyclic voltammetry with mediators. Still, in these set of voltammograms is observed the working principle of PQQ-enzymes (Figures 36, 37 and 38). In the presence of PQQ-ADH/ALDH is visible the oxidation peak, due to enzymatic catalysis in relation to the small peak of working electrode without cells or enzymes i.e., in relation to control voltammograms.

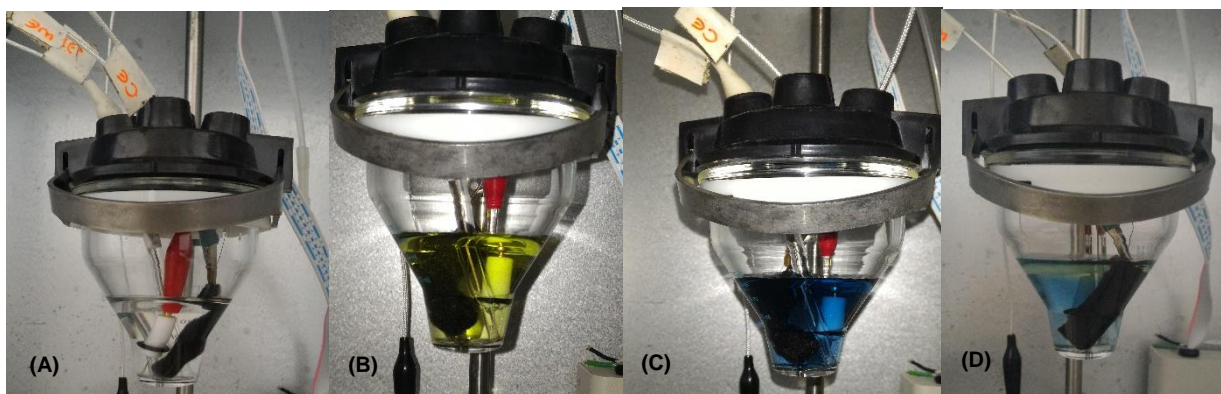


Figure 35: MET on Cyclic Voltammetry - (A): Electrochemical Cell compartment with 0.2 M Tris-HCl buffer pH 7.5 and 1 mM CaCl_2 ; (B): PMS before reaction; (C): PMS and DCPIP. (D): Electrochemical Cell with 0.2 M Tris-HCl buffer pH 7.5, 1 mM CaCl_2 , PMS, DCPIP in end of reaction. It is possible to see a shift from dark blue to light blue at end of the reaction and up thin layer of PMS in the solution.

The voltammograms obtained with working electrode with cells (**Cells**) in presence of ethanol and mediators (MET mechanism) show a good performance when compared with control (Figure 36). Higher ethanol concentrations resulted in peaks with higher oxidation currents mainly at ~ -0.19 V and higher reduction current at ~ -0.2 V. However, there is also a shift of the peak from -0.15 V to -0.19 V for control working electrode and working electrode with cells, respectively for ethanol substrate in the reaction media. Furthermore, it is visible a second characteristic peak of current at ~ 0.6 V as already observed for DET mechanism experiments.

The peaks at -0.19 V involving the mediators the current increase with ethanol concentration from 2.20×10^{-3} A, 2.83×10^{-3} A and 2.98×10^{-3} A for **Cells**, **Cells 5 mM** ethanol and **Cells 10 mM** ethanol, respectively. This increase in the oxidation current with MET mechanism corresponds about 10 times higher than those one obtained with DET mechanism. Also, it can be observed in second peak of reduction current at -0.2 V that also increase with ethanol concentration from -2.45×10^{-3} A, -3.14×10^{-3} A and -3.43×10^{-3} A for **Cells**, **Cells 5 mM** ethanol and **Cells 10 mM** ethanol, respectively.

But in contrast with it was observed in DET mechanism now the third peak at ~ 0.6 V the oxidation current increases with ethanol concentration from 1.32×10^{-4} A, 1.73×10^{-4} A and 4.20×10^{-4} A for **Cells**, **Cells 5 mM** ethanol and **Cells 10 mM** ethanol, respectively.

As observed previously for biocatalysts of whole *Gluconobacter* cells (**Cells**), the working electrode with enzyme from the supernatant (**supernatant**) shows a good performance in MET too. There were two peaks, one due to oxidation current at -0.19 V and other of reduction current at -0.2 V (Figure 37). In first peak, - 0.19 V, it is possible to see that the oxidation current increase with ethanol concentration from 1.99×10^{-3} A, 2.17×10^{-3} A, 2.32×10^{-3} A and 2.73×10^{-3} A for **supernatant**, **supernatant 5 mM ethanol**, **supernatant 10 mM ethanol**, **supernatant 20 mM ethanol**, respectively. The smaller values of oxidation current obtained with working electrodes with enzymes in relation to those one obtained with the cells is due to lower PQQ-enzyme activity extracted to the supernatant.

Similarly, for second peak at -0.2 V the reduction current increase with ethanol concentration from -2.25×10^{-3} A, -2.37×10^{-3} A, -2.44×10^{-3} A and -2.88×10^{-3} A **supernatant**, **supernatant 5 mM ethanol**, **supernatant 10 mM ethanol**, **supernatant 20 mM ethanol**, respectively.

Similar to it was already observed for working electrode with enzymes in DET mechanism now for MET it is not also possible correlate the oxidation current at -0.6V with ethanol concentration as values aren't coherent, i.e., 3.40×10^{-4} A, 3.35×10^{-4} A, 3.05×10^{-4} A and 3.08×10^{-4} A **supernatant**, **supernatant 5 mM ethanol**, **supernatant 10 mM ethanol**, **supernatant 20 mM ethanol**, respectively.

MET (Mediator Electron Transference) with glycerol

Finally, it was tested the working electrodes with immobilized cells (**cells**) on the oxidation of glycerol (Figure 38). The working electrode with **Cells** didn't provide a better performance in relation to the similar experiment but with ethanol.

Similar to what occurred previously with **Cells** with ethanol, the working electrodes with cells and glycerol there are 2 peaks of current, in -0.19 V and -0.2 V. In first peak, -0.14 V, it is possible to see that the oxidation current increase with glycerol from 1.25×10^{-3} A, 1.56×10^{-3} A and 1.62×10^{-3} A for **Cells**, **Cells 5 mM glycerol** and **Cells 10 mM glycerol**, respectively. Similarly, it can be observed in second peak at -0.2 V, that reduction current decreases with glycerol from -1.30×10^{-3} A, -1.91×10^{-3} A and -2.10×10^{-3} A for **Cells**, **Cells 5 mM glycerol** and **Cells 10 mM glycerol**, respectively.

Also in this case, it presented a second peak at ~ 0.6 V but it is not very well known the origin. However, the oxidation current is smaller with glycerol as it is already observed for ethanol experiments and in this case from 3.52×10^{-4} A, 3.37×10^{-4} A and 3.48×10^{-4} A for **Cells**, **Cells 5 mM glycerol** and **Cells 10 mM glycerol**, respectively.

As referred in DET mechanism, other authors, also performed cyclic voltammetry by MET mechanism in same conditions than DET [75]. The enzymes immobilization at working electrode was made with (TBAB)- modified Nafion on a glass carbon surface, and the experiments were also conducted with PMS and DCPIP. Also, MET mechanism provide good results. It is clear two peaks, corresponding to oxidation and reduction of enzymes, - 0.15 and - 0.22 V respectively, where it is possible to see a current increase as ethanol concentration was added. In our experiments the peaks

of current occurred at -0.19 and -0.20 V for cells with ethanol and glycerol and -0.19 and -0.12 V for supernatant.

In our reference article, the working electrode presented higher values of current density, 110 $\mu\text{A}/\text{cm}^2$, for a TBAB- modified Nafion electrode [75]. In our experiments were obtained values of current density of 131, 105 and 74 $\mu\text{A}/\text{cm}^2$, for cells with 10 mM ethanol, supernatant with 10 mM ethanol and cells with 10 mM glycerol. Based in these values, it is possible to conclude that MET mechanisms have a good performance in our experiments, better than working electrode used in MET by reference article [75].

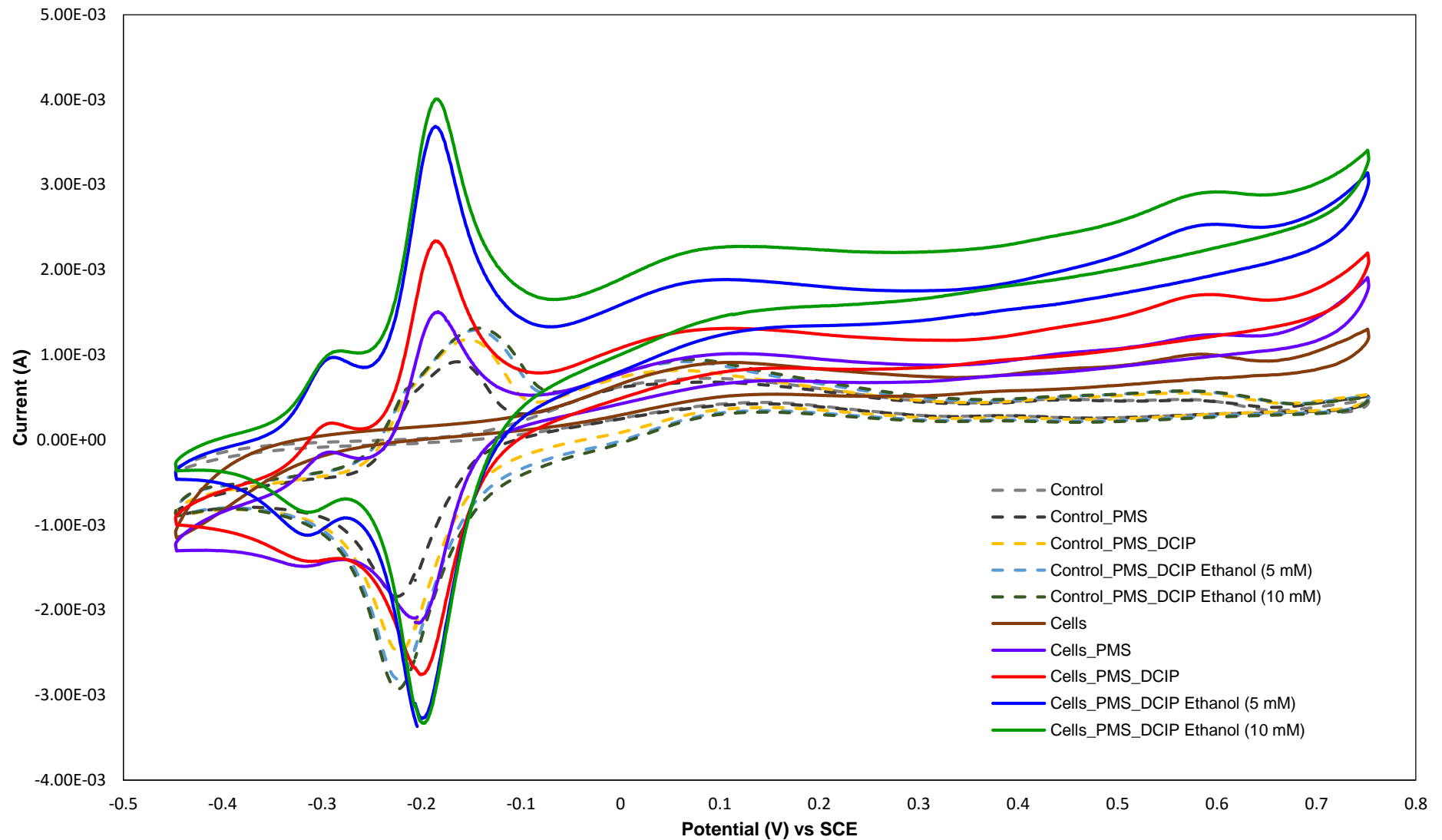


Figure 36: Representative cyclic voltammograms with the working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from original cells, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface. Also, it is presented the control of working electrode, i.e., cells free. The assays were made based of MET mechanism, in Tris-HCl buffer and CaCl_2 , 35 μM DCPIP and 60 μM PMS, with 5 / 10 mM of ethanol, pH 7.5 at voltage rate of 0.010 $\text{V} \cdot \text{s}^{-1}$.

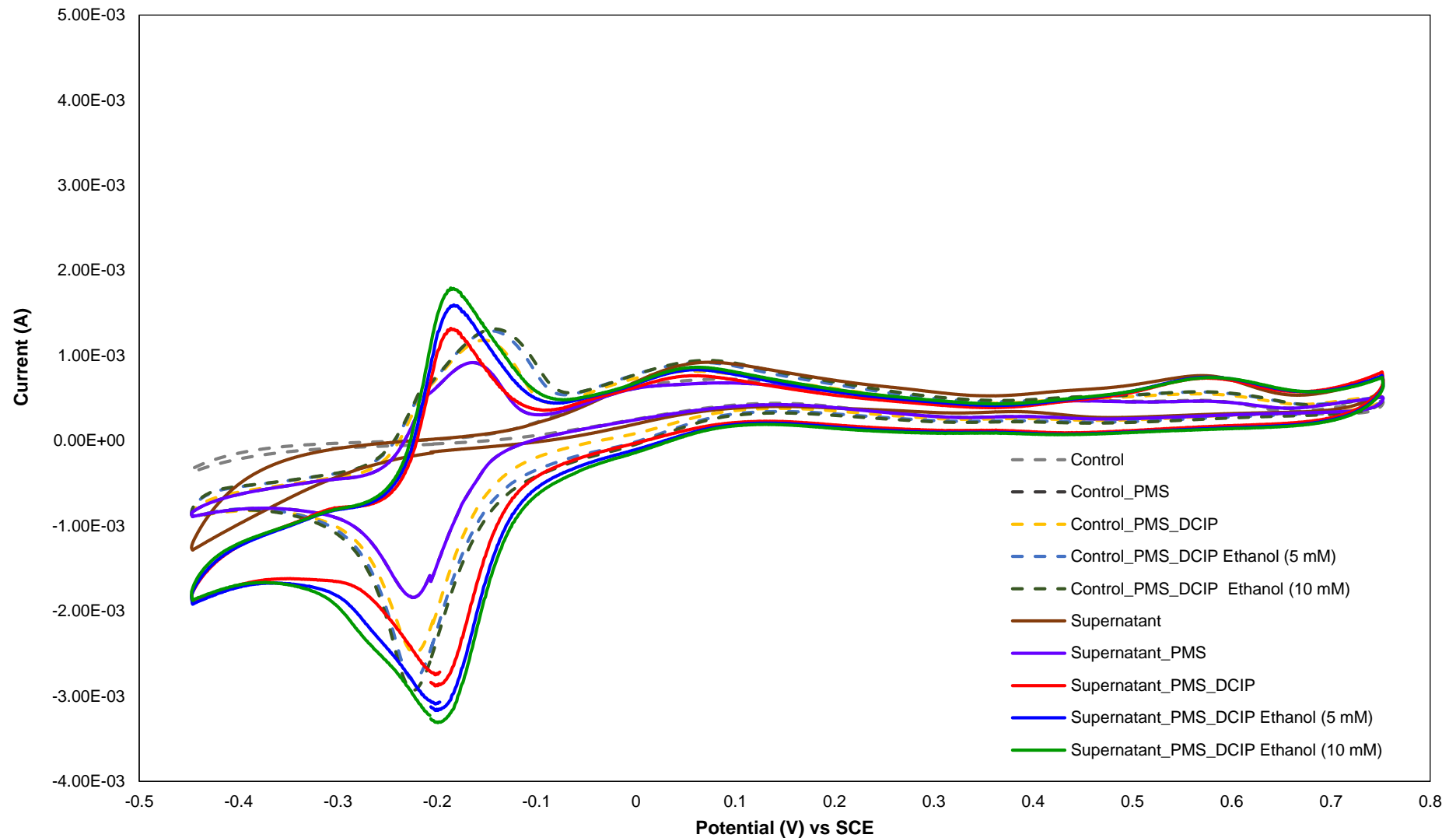


Figure 37: Representative cyclic voltammograms with working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from supernatant, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface. Also, it is presented the control of working electrode, i.e., cells free. The assays were made based of MET mechanism, in Tris-HCl buffer and CaCl_2 , 35 μM DCPIP and 60 μM PMS, with 5 / 10 mM of ethanol, pH 7.5 at voltage rate of 0.010 $\text{V} \cdot \text{s}^{-1}$.

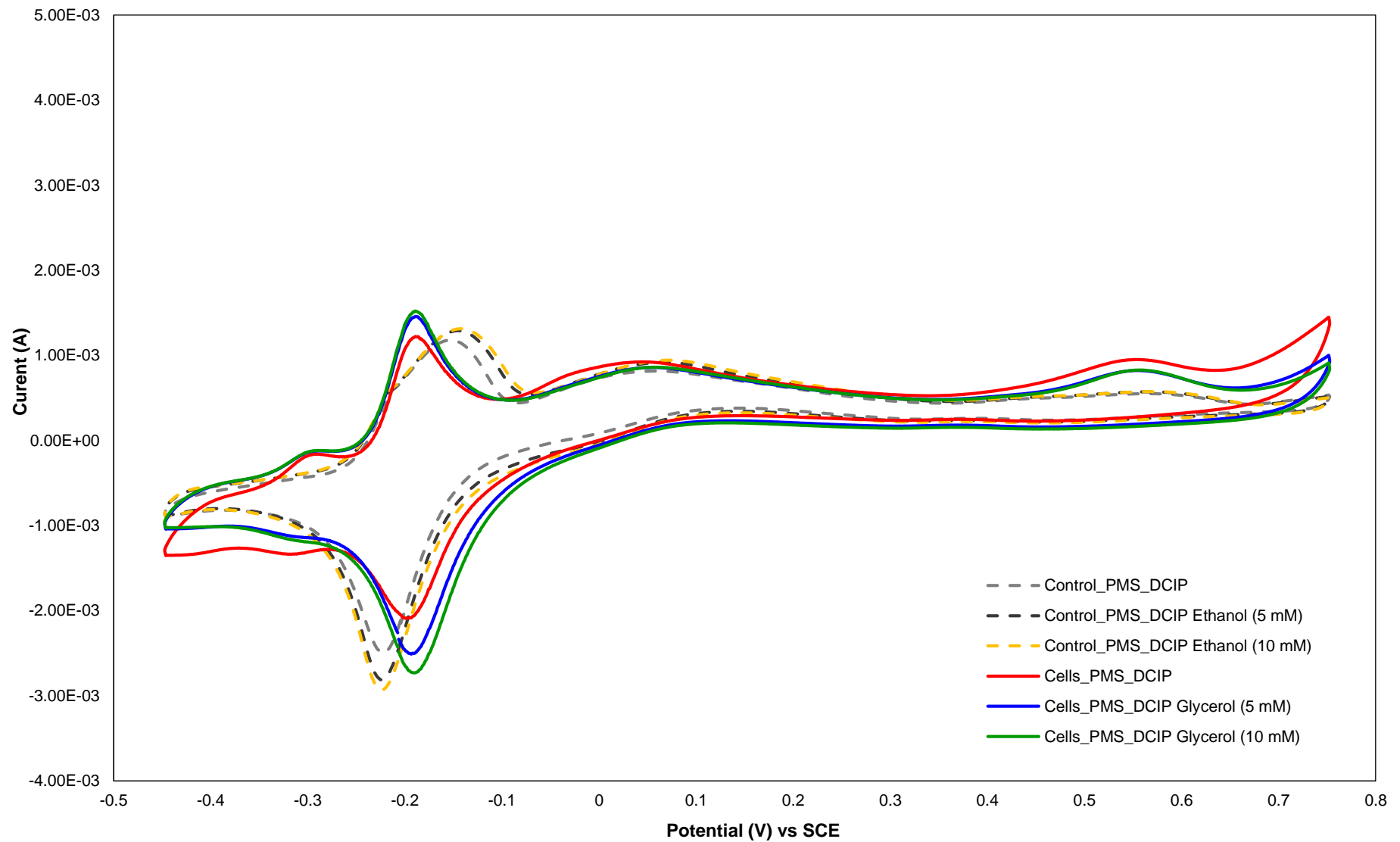


Figure 38: Representative cyclic voltammograms with working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from original cells, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface. Also, it is presented the control of working electrode, i.e., cells free. The assays were made based of MET mechanism, in Tris-HCl buffer and CaCl_2 , 35 μM DCPIP and 60 μM PMS, with 5 / 10 mM of glycerol, pH 7.5 at voltage rate of 0.010 $\text{V} \cdot \text{s}^{-1}$.

4.4.3. CHRONOAMPEROMETRY

Chronoamperometry assay was based in last results of MET mechanism, for working electrode with cells (**Cells**) and the two substrates, ethanol and glycerol. For this characterization of assay method was fixed an oxidation potential of - 0.19 V and added successive amounts of 2 mM ethanol/glycerol. Therefore, in Figures 39 and 41 show clearly to achieve a stable plateau of current obtained for each substrate concentration and the current increase for consecutive amount of substrate added into the electrolyte.

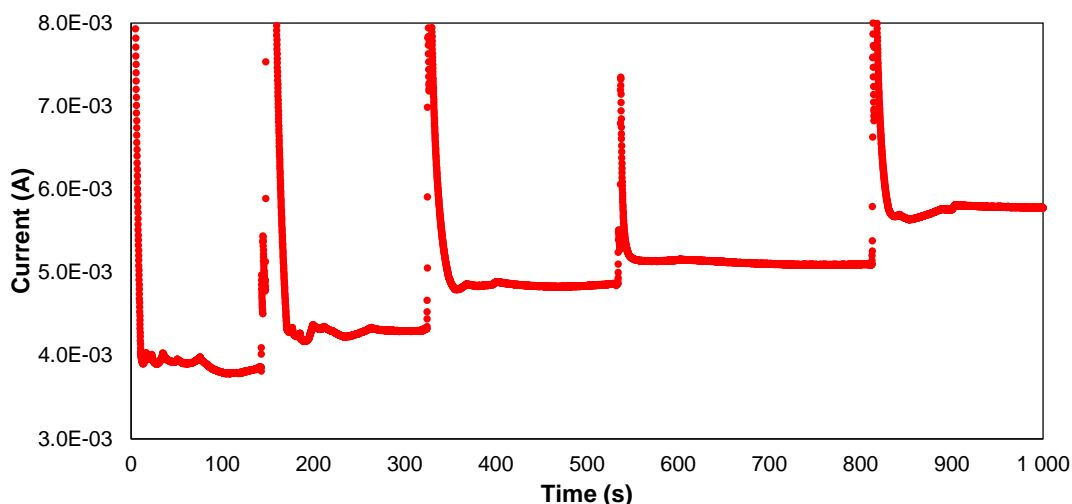


Figure 39: Chronoamperometric assay performed with a working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from cells, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface, at a fixed oxidation potential, -0.19, by gradually increasing the concentration the concentration of ethanol in the electrolyte solution in 0.2 M Tris-HCl buffer and CaCl_2 , pH 7.5. The assays were made based on MET mechanism with 12 mM DCPIP and 20 mM PMS.

These chronoamperometry assays show also a linear increase of current with ethanol concentration from 3.9×10^{-3} A to 5.8×10^{-3} A until to achieve a maximum of current for ethanol concentration (10 mM) (Figure 39). After that ethanol concentration (between 12 and 20 mM in the electrolyte solution) there was consecutive reduction of current values when more substrate was added into the electrolyte solution.

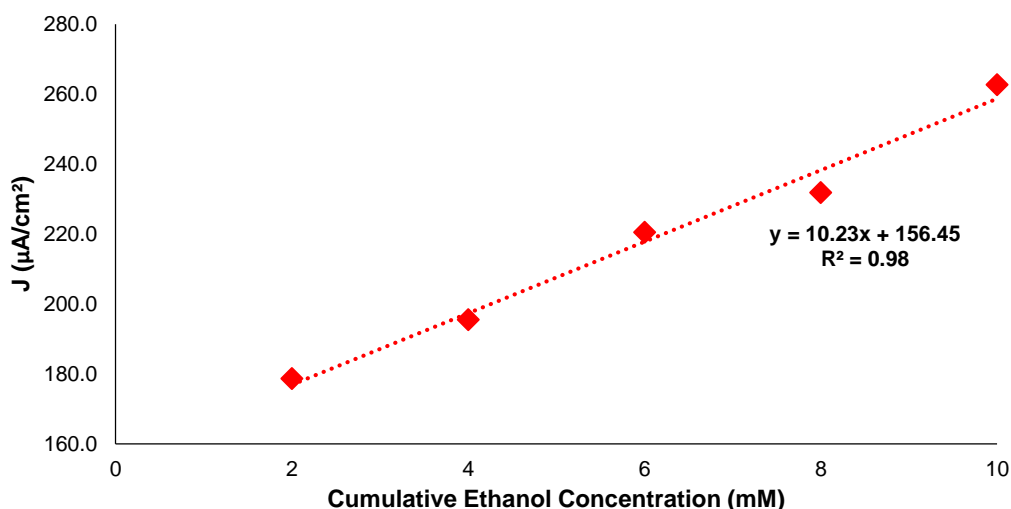


Figure 40: Representation of relation of current per electrode surface area by gradually increasing of the ethanol concentration, from 2 mM to 10 mM, in the electrolyte solution with mediators and working electrode with (**cells**) at - 0.19 V.

Also, it is possible to see in Figure 40, a linear increase of current density as ethanol concentration was added. It was obtained a maximal current density of $262 \mu\text{A}/\text{cm}^2$, however in a reference article it was obtained $1200 \mu\text{A}/\text{cm}^2$, although with a different working electrode [75].

Similar experiment was carried out with working electrode cells (**Cells**) but now for glycerol. Figure 41, shows an increase of current at -0.19 V with consecutive additions of glycerol from $4.7 \times 10^{-5} \text{ A}$ to $4.2 \times 10^{-4} \text{ A}$. Also, in chronoamperometry assays for glycerol was possible to observe a linear increase of current as glycerol concentration was added, in Figure 42, but in this case it was not observed maximum value and reduction of the current even for higher glycerol concentrations in comparison what it was observed with ethanol.

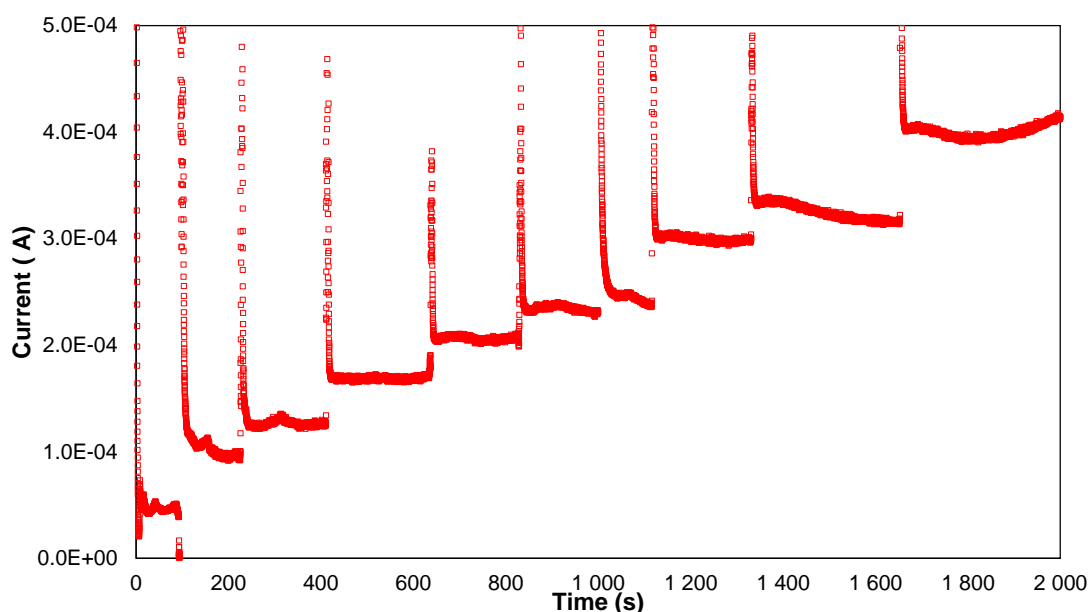


Figure 41: Chronoamperometric assay performed with a working electrode containing both PQQ- dependent dehydrogenases, ADH and ALDH, from cells, immobilized with 0.5% of Glutaraldehyde on a carbon felt surface, at a fixed oxidation potential, -0.15 , by gradually increasing the concentration of glycerol in the electrolyte solution in 0.2 M Tris-HCl buffer and CaCl_2 , pH 7.5 at $0.010 \text{ V} \cdot \text{s}^{-1}$.

The comparison of these two group of experiments of chronoamperometry assays with working electrode of cells (**Cells**) the current range measured is higher $5.8 \times 10^{-3} \text{ A}$ and $4.2 \times 10^{-4} \text{ A}$ for ethanol and glycerol, respectively, i.e., the values of current for ethanol is around 10 times bigger than those one obtained with glycerol. It was obtained a maximal current density of $19 \mu\text{A}/\text{cm}^2$, really smaller than the same experiment with ethanol.

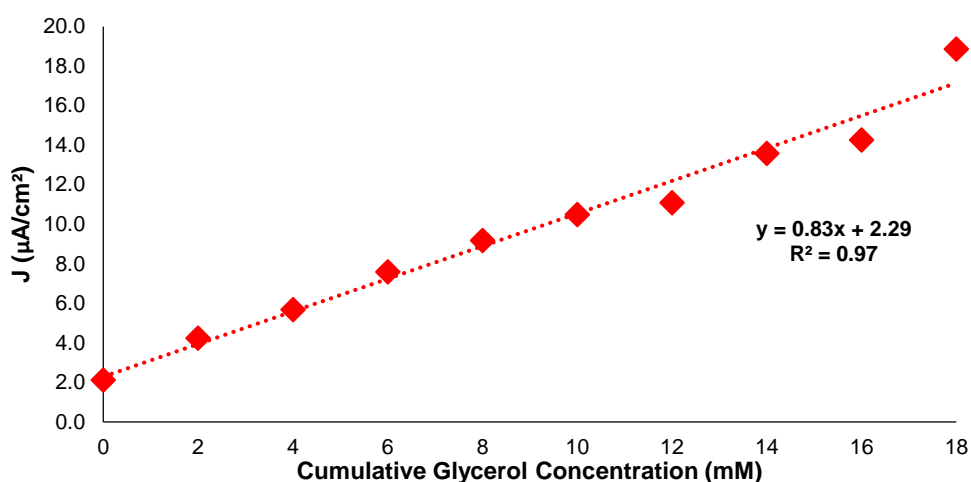


Figure 42: Representation of relation of current per electrode surface area by gradually increasing of the glycerol concentration, from 2 mM to 18 mM, in the electrolyte solution with mediators and working electrode with cells (Cells) at -0.19 V.

4.5. BIOFUEL CELL – ENZYMATIC POWER BANK

4.5.1. DET MECHANISM IN POWER PRODUCTION

The energy production with the assembly Biofuel Cell was assayed for DET and MET mechanisms. This can be achieved by two electrons transfer mechanisms that can occur in oxidation of substrates in Bioanode through the PQQ-enzymes in whole *Gluconobacter* sp.33 cells.

As it was observed in cyclic voltammetry, DET didn't show a good performance in this Biofuel Cell. Two experiments were performed in order to evaluate DET mechanism in the Bioanode of the assembled Biofuel Cell (Figure 43). It is clear that occur production of current. In spite of, this current is really small but consequently prove that occur electrons transfer produced by the whole *Gluconobacter* cells and collected directly by the carbon felt.

Nevertheless, it is possible to see a current production after ethanol addition. Cells produced 4 ± 4 μ A of current intensity, in terms of average value, while the maximum values of current intensity were around 17, 11 and 7 μ A. In control experiments isn't observe significant current variation when ethanol was added as the current intensity was residual along of all experiment (Figure 43).

Based on last results, other experiments were performed in order to improve electrons transference in this BioFuel Cell by changing the circulating electrolyte solution on the cathodic chamber to potassium permanganate solution 0.04 mM, instead of water with air bubbling.

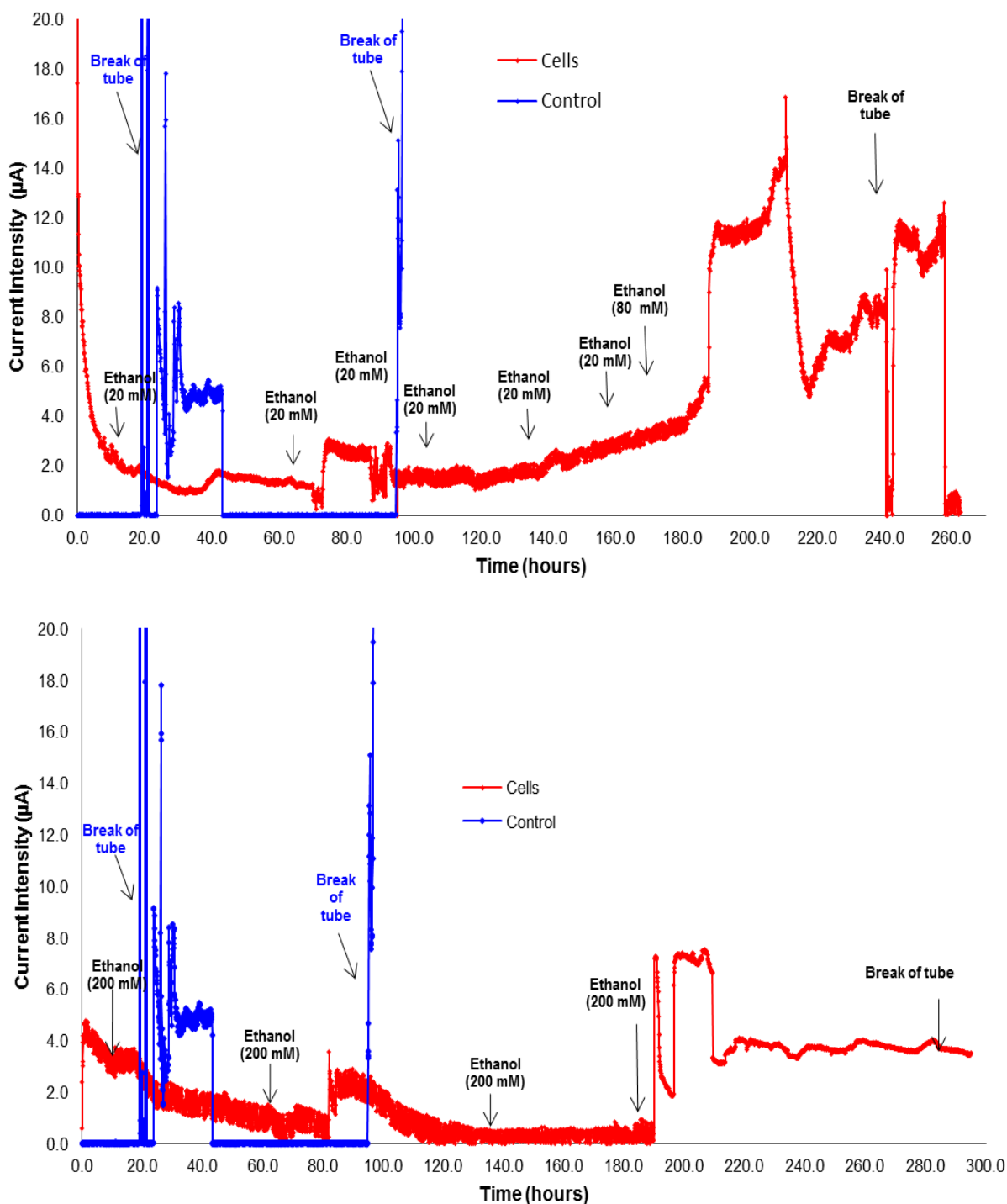


Figure 43 : Current Intensity produced in Bioanode of BFC, over DET mechanism. The anodic chamber contains 100 mL of potassium phosphate buffer 50 mM, pH 7.3 and it was added ethanol (20 and 80 mM), along the time. The cathodic chamber was used water saturated with air bubbling. In control experiments was performed the same way but carbon felt without cells

According this experiment it was possible to verify that occur electrons transference too and there was an improve of the current production when ethanol was added (Figure 44). The current produced in this experiment was $5 \pm 3 \mu\text{A}$ of current intensity in terms of average value, and sometimes maximum values of current intensity around 17 and 15 μA .

Still, it is possible to observe the production of current increase of ethanol concentration with first additions of ethanol, but over the time and for higher ethanol concentrations begins to decrease (Figure 45). This reduction of current intensity can be due to several factors but mainly to inhibition or deactivation of PQQ-enzymes inside of *Gluconobacter* cells due to ethanol toxicity or accumulation of products of the redox reaction such as aldehydes or acids that change the electrolyte composition for higher ethanol concentration.

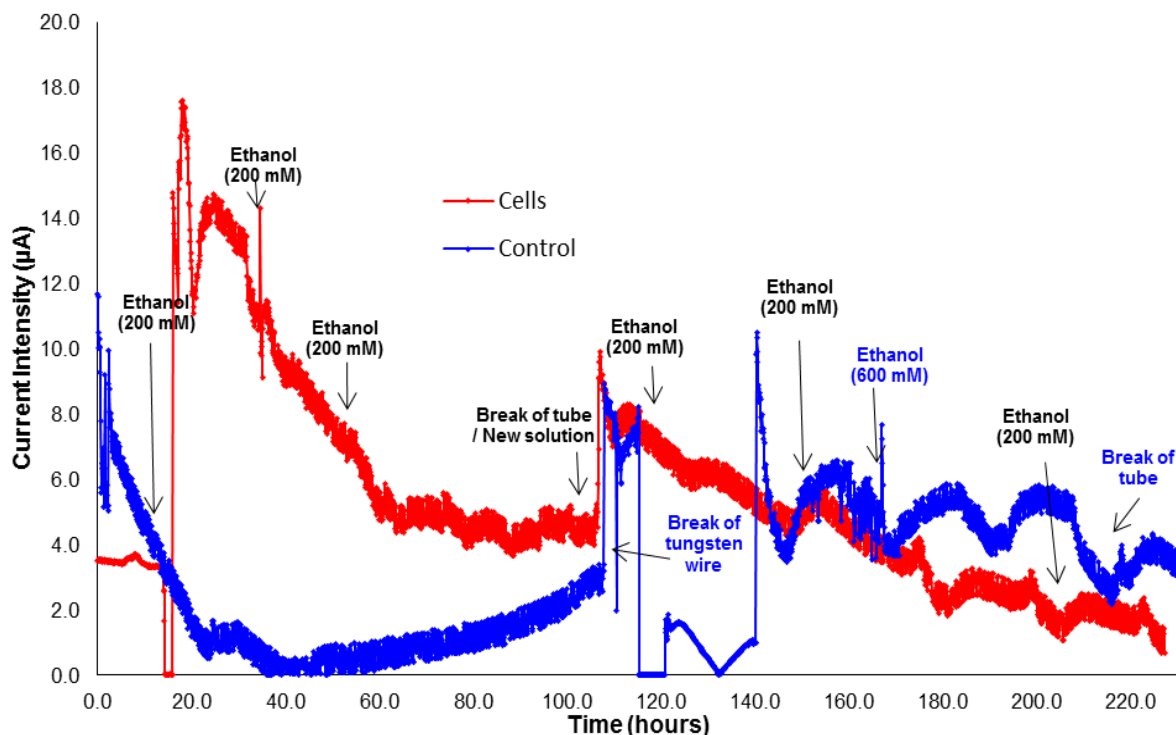


Figure 44: Current Intensity produced by Bioanode of BioFuel Cell, over DET mechanism. The biofuel cell contained 100 mL of potassium phosphate buffer 50 mM, pH 7.3 and it was added ethanol (200 mM) along time. In the cathodic chamber was used potassium permanganate 0.04 mM, as circulation electrolyte solution. In control experiments was performed the same way but without cells.

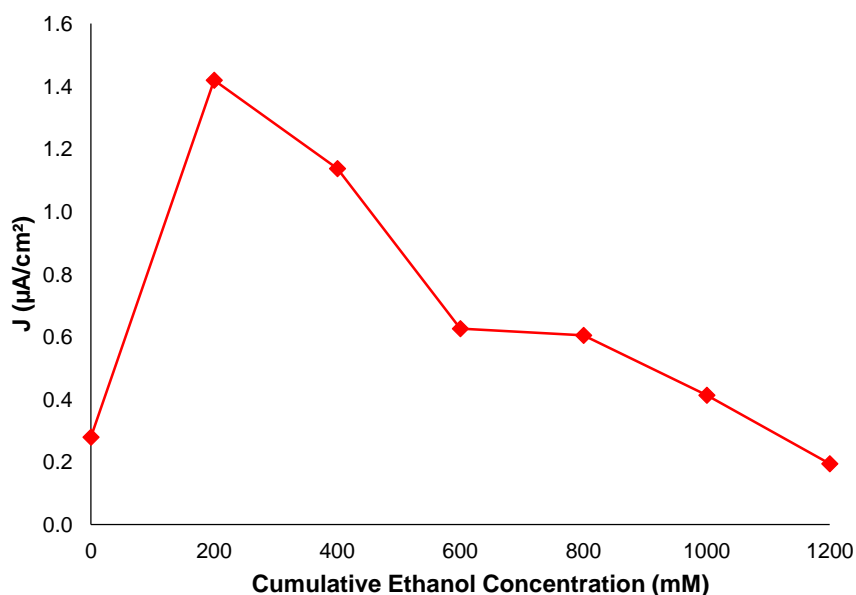


Figure 45 : Range of current intensity (μA) by anode surface area versus ethanol concentration (mM). The biofuel cell contained 100 mL of potassium phosphate buffer 50 mM, pH 7.3 and it was added ethanol (200 mM) along time. To cathode chamber was used potassium permanganate 0.04 mM, as circulation electrolyte solution.

Comparing to other work, the maximal current density obtained was $3500 \mu\text{A}/\text{cm}^2$ [43], and in this biofuel cell only $1.4 \mu\text{A}/\text{cm}^2$. Still, it is important to refer that concentration levels aren't accurate, because it was expected that enzymes consumed the substrate and therefore occurred direct electron transference.

4.5.2. MET MECHANISM IN POWER PRODUCTION

The MET mechanism in this BioFuel Cell for ethanol and glycerol, as substrates was evaluated.

As it was observed in cyclic voltammetry, MET mechanism was proved to occur with ethanol as substrate for this Bioanode based on whole *Gluconobacter* cells immobilized in carbon felt. The PQQ-enzymes in whole cells can transfer electrons through the mediators and generate power as can see in Figure 46.

Current of $25 \pm 20 \mu\text{A}$ in terms of average value, and sometimes maximum values of around 77, 50 and $35 \mu\text{A}$ were produced. However, the current values obtain with this BioFuel Cell are smaller than it will be expected when comparing for references.

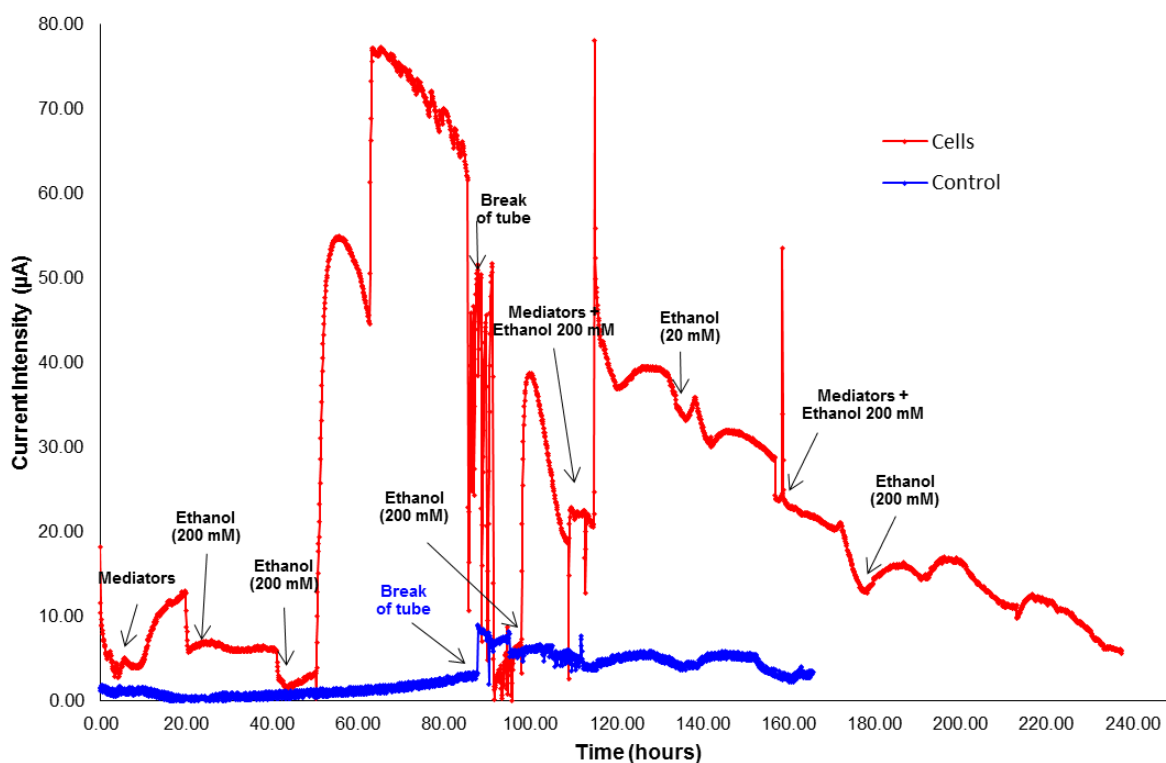


Figure 46: Current Intensity produced by cells, over MET mechanism. The biofuel cell contained 100 mL of potassium phosphate buffer 50 mM, pH 7.3, $35 \mu\text{M}$ PMS, $60 \mu\text{M}$ DCPIP and it was added ethanol (200 mM) along time. To cathode chamber was used potassium permanganate 0.04 mM, as circulation electrolyte solution. In control experiments was performed the same way but without cells.

Still, it is possible to observe that the production of current increase with ethanol concentration in first additions but over the time and for higher ethanol concentrations begins to decrease. This reduction can be due to several factors previously referred previously (Figure 47). Comparing to other work, the maximal current density obtained was 3500 $\mu\text{A}/\text{cm}^2$ [43], and in this experiment only 6.1 $\mu\text{A}/\text{cm}^2$.

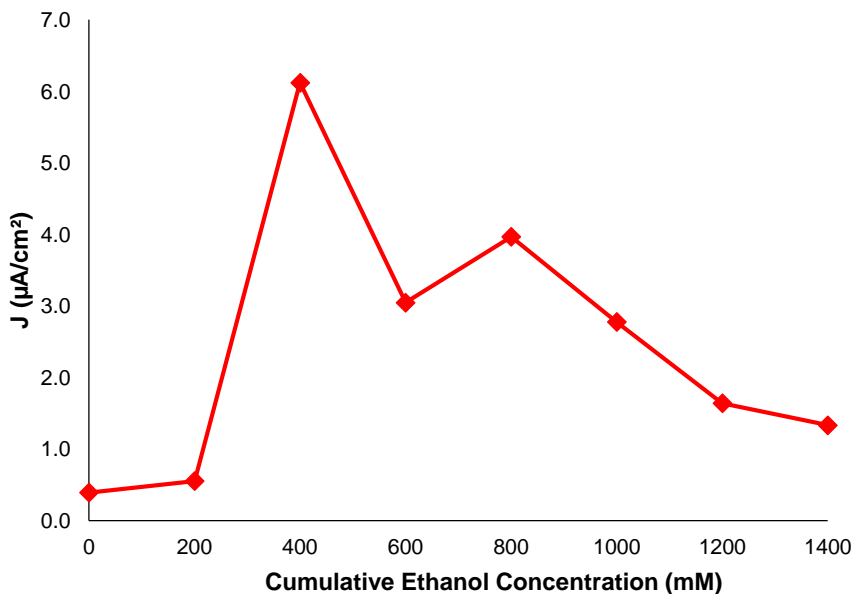


Figure 47: Range of current intensity (μA) by cathode surface area versus ethanol concentration (mM). The biofuel cell contained 100 mL of potassium phosphate buffer 50 mM, pH 7.3, 35 μM PMS, 60 μM DCPIP and it was added ethanol (200 mM) along time. To cathode chamber was used potassium permanganate 0.04 mM, as circulation electrolyte solution.

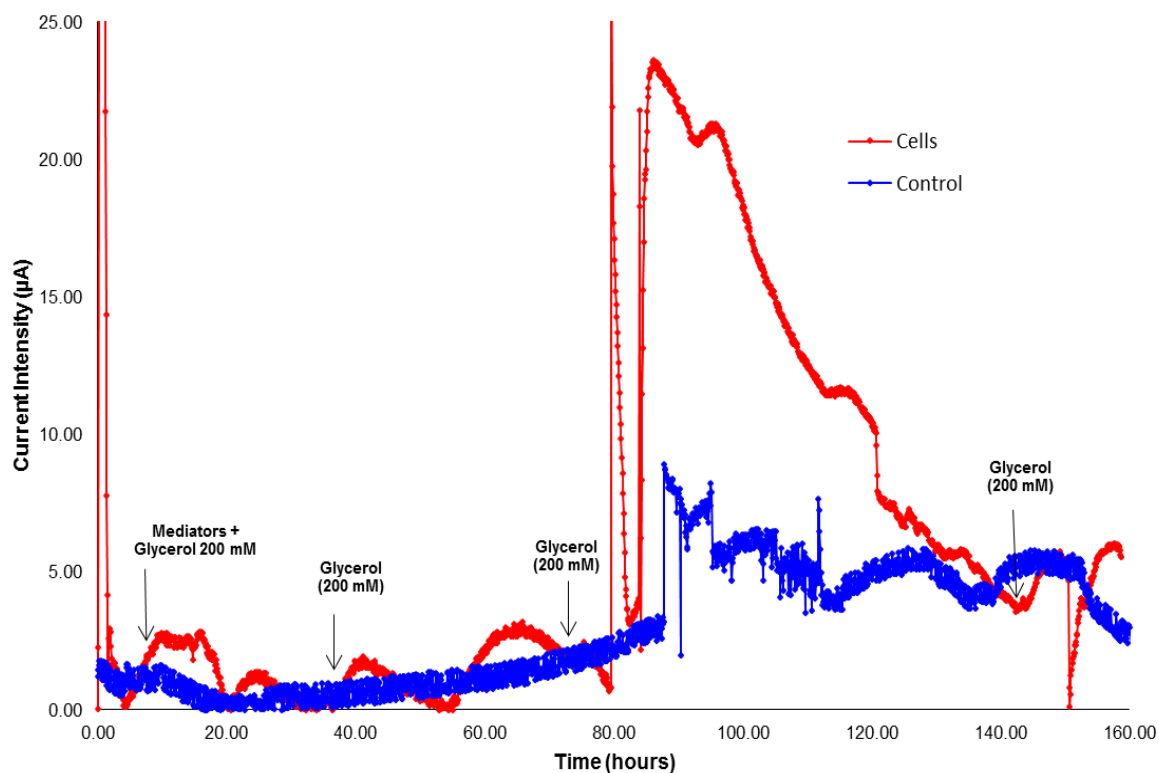


Figure 48: Current Intensity produced by cells, over MET mechanism. The biofuel cell contained 100 mL of potassium phosphate buffer 50 mM, pH 7.3, 35 μM PMS, 60 μM DCPIP and it was added glycerol (200 mM) along time. To cathode chamber was used potassium permanganate 0.04 mM, as circulation solution. In control experiments was performed the same way but without cells.

Finally, it was performed the assay of MET mechanism for this BioFuel Cells but now using glycerol as substrate. As it was observed in the cyclic voltammetry studies the result of power production was also smaller than those one obtained with ethanol (Figure 48). However, it is possible to see a current increase as glycerol concentration was added with average value of $6 \pm 7 \mu\text{A}$ of current intensity and sometimes maximum values around 23 and 21 μA .

From Figure 49 is possible to observe the production of current increase with accumulative glycerol concentration but the current intensity only increase after two additions of glycerol (200mM). Comparing to other work, the maximal current density obtained was $3500 \mu\text{A}/\text{cm}^2$ [43] with glycerol as substrate and with PQQ-ADH and PQQ-ALDH, in our biofuel cell only $1.9 \mu\text{A}/\text{cm}^2$.

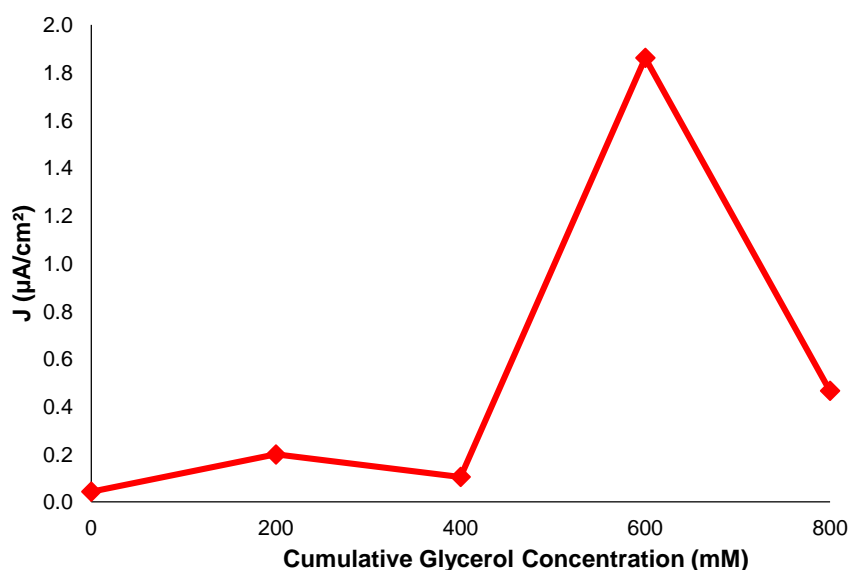


Figure 49: Range of current intensity (μA) by anode surface area versus glycerol concentration (mM). The biofuel cell contained 100 mL of potassium phosphate buffer 50 mM, pH 7.3, 35 μM PMS, 60 μM DCPIP and it was added ethanol (200 mM) along time. To cathode chamber was used potassium permanganate 0.04 mM, as circulation solution.

Bellow, in table 7, it is present a resume of all current values obtained in all experiments performed in a BFC. It possible to observed all average values of current intensity generated as well as, the maximal current density obtained in bioanode of BFC. It's clear that cells with ethanol as fuel by MET have the best power generation.

Table 7: Values of Current Intensity and Maximal Current Density obtained in all experiments of BFC's, through *Gluconobacter* sp. 33 cells by DET and MET mechanisms with ethanol and glycerol as fuels.

	Average Values of Current Intensity obtained (μA)	Maximal Current Density obtained ($\mu\text{A}/\text{cm}^2$)
DET Mechanism		
Ethanol (cathodic electrolyte solution: H_2O / Air)	4 ± 4	-
Ethanol (cathodic electrolyte solution: Potassium permanganate 0.04 mM)	5 ± 3	1.4
MET Mechanism		
Ethanol (cathodic electrolyte solution: Potassium permanganate 0.04 mM)	25 ± 20	6.1
Glycerol (cathodic electrolyte solution: Potassium permanganate 0.04 mM)	6 ± 7	1.9

4.5.3. POWER DENSITY BY SURFACE AREA

In recent years, authors have referred that the performance of Bioanode can be improved by the use of high surface materials, such as carbon nanotubes [43]. Power density versus current intensity were calculated which provide the power curves of the Biofuel Cell assembled in this work (Figure 50).

In terms values, the maximal power density produced by surface area was 4.6×10^{-7} and 1.3×10^{-7} W/cm², for cells with ethanol and glycerol, respectively. In other study, with these enzymes and glycerol as substrate were achieved 4.5×10^{-4} W/cm² [43]. These values can be improved by increase of surface area of bioanode or change of some materials that promote the electrons transference.

Based in Figure 50, it is possible to conclude that only BioFuel Cells with ethanol and glycerol, over MET mechanisms, present a directly increase of power as increase of current intensity, i.e., power production by surface area is really higher than control experiment.

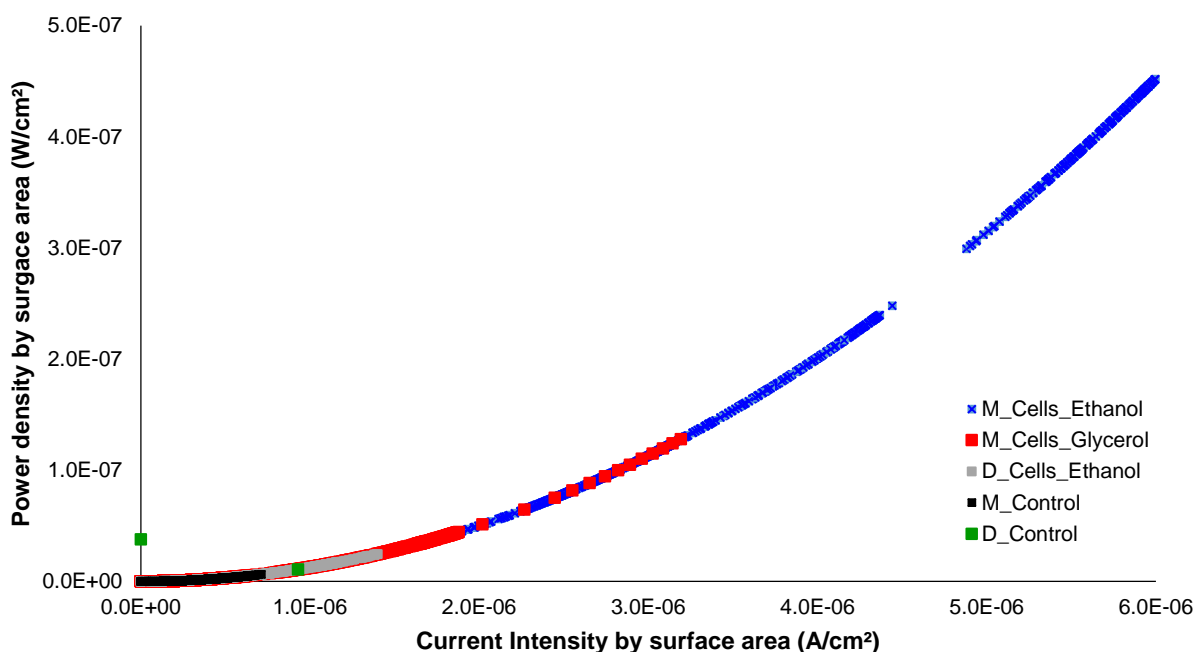


Figure 50: Comparison of power curves generated in a Biofuel cell. Only it was presented the experiments that had as circulation electrolyte solution of potassium permanganate 0.04 mM in cathodic chamber.

CHAPTER 5: CONCLUSIONS AND FUTURE PERSPECTIVES

The generation of power from *Gluconobacter sp.* 33 cells in a BioFuel Cell, the goal of this dissertation, was achieved by mediator electron transference (MET).

The growth of *Gluconobacter sp.* 33 was obtained in all fermentation media tested, however the best growth was reached in a different medium that was indicated in references, YPM + GLY instead BM. Also, in this medium *Gluconobacter sp.* 33 specific growth rate was determined as $\mu = 0.1187 \text{ h}^{-1}$, slightly larger than obtained in those references, but the inoculum used wasn't commercial. Still, it was possible determine a correlation between dry cell concentration in (mg/mL) and OD values with several dilutions, which is given by: $Y = 1.208 X + 0.032$, that can be used in future in order to estimate the amount of cells produced in each fermentation.

In the future, the BM and GYC media, should be studied to improve the production of enzymes. Starting from BM medium, it will be tested exactly all compounds that were used for enzymes production in references. In this work, the ammonium phosphate was changed to di-ammonium hydrogen phosphate because it wasn't available. In relation to GYC medium, the calcium carbonate can be change to other compound, because it didn't dissolve in medium or improve and adjust the amount of this compound in medium.

PQQ-ADH / ALDH had considerable levels of enzymatic activity, in particular, they have 8.0 and 11.4 U/mL, respectively. Still, it is observed that the enzymes are stable over the time, i.e., kept viable till 6 weeks of storage at 4 °C. As future work is recommended to determine the amount of protein present in order to obtain a specific enzymatic activity (U/mg).

Still, one factor that it is important to refer that *Gluconobacter sp.* 33 grew in YPM media supplemented with glycerol, but the induction of enzymes production in these media with *Gluconobacter sp.* 33 is not presented [79,80]. Thus, based in results obtained it is possible to say that occur growth of *Gluconobacter sp.* 33 in YPM medium supplemented by glycerol and induction of PQQ-ADH and PQQ-ALDH, because there was an increase of enzymatic activity levels in these media under these conditions. This is an important point in this dissertation since its innovative character can lead to new developments and investigations in this field.

In order to have more enzymatic activity, studies were performed, such as: cell permeabilization, extraction and purification of enzymes from supernatant. In cells permeabilization were again observed that YPM+GLY is the medium with most enzymatic activity. Also, it was clear that enzymes were losing enzymatic activity with treatment of cells permeabilization. Several step of cell permeabilization were tested, such as: the effect of sonication, storage, deoxycholate and lysozyme proportion in order to understand that interfere in the loose of enzymatic activity.

The sonication proved that occurred loss of enzymatic activity during this step, and for its was removed to the process. The cells showed more enzymatic activity when stored in a frozen at - 20 °C, although the difference when stored at 4 °C in a fridge is really smaller. This fact didn't affect this work because in a BioFuel Cell only were used fresh cell. In relation to deoxycholate and lysozyme proportion in cell permeabilization, 0.5 % of cells permeabilization was the result with more enzymatic activity, however, these values are really smaller when compared to original cells. In future studies, the proportion of this compounds need to be explored and adjusted. As the enzymes are localized at cytoplasmic membrane and the sonication process and cell permeabilization occur by rupture of cytoplasmic membrane need to be improve the time of process, energy (sonication), amount of compounds used, as well as control of temperature process.

Still, from supernatant of BM was performed the extraction and purification of PQQ-ADH / ALDH, however its didn't have significant levels of enzymatic activity, as happened in cells of BM. In future should be optimized the time of each compound in purification process or even so, change the compounds, because, as PQQ-ADH /ALDH are membrane bound enzymes require a buffer that solubilize the enzymes instead affect the ionizable groups.

In electrochemical measurements the working electrode show a good performance. It is was proved that enzymes / cells remained immobilized at working electrode and viable to use forward. Then, in cyclic voltammetry DET mechanism have a poor performance, almost all voltammograms obtained have peaks of current (except to cells free) however the current increase as ethanol concentration was added it was really smaller. On the contrary, MET mechanism provided really good results for all experiments, it was clear a current increase as ethanol / glycerol concentration was added. In terms of current density of working electrode achieved 131, 105 and 74 $\mu\text{A}/\text{cm}^2$, for cells with 10 mM ethanol, supernatant with 10 mM ethanol and cells with 10 mM glycerol, all through MET mechanism. If compared with our reference article, it was reach more 21 $\mu\text{A}/\text{cm}^2$ in carbon felt electrode than TBAB- modified Nafion electrode (110 $\mu\text{A}/\text{cm}^2$) [75].

The chronoamperometric experiments proved the concept of power production by *Gluconobacter sp.* 33 cells by increase of current as substrate concentration was added. Chronoamperometry assays show a linear increase of current with substrate concentration from 3.9×10^{-3} A to 5.8×10^{-3} A and from 4.7×10^{-5} A to 4.2×10^{-4} A, for cells with ethanol and glycerol, respectively.

In the future, the compounds of biofuel cell need to be adjusted. The tungsten wire broke several times, as well as the tube of cathode solution. Also, in MET mechanism to power production, in the layer of carbon felt at bioanode occurred a little degradation of layer by mediators. It is recommended an alternative search of these materials in order to improve the electron transference, for example, a Toray paper is referenced by authors in assembly of biofuel cell like it [43].

In DET mechanism in power production were performed with two solutions in cathode: water with air bubbling and potassium permanganate 0.04 mM. In both assays, it was clear that occurred production of current, but a little more with potassium permanganate 0.04 mM as cathodic solution. However, this current is really small but consequently prove that occur electrons transfer produced by the whole *Gluconobacter* cells and collected directly by the carbon felt.

In the future, a new BFC's structure should be tested or some materials' changes should be done in order to improve the power production through DET mechanism.

MET mechanism in BioFuel cell provide the best results of power production. It was clear that occurred production of current as ethanol / glycerol concentration was added. In terms of average values were produced $25 \pm 20 \mu\text{A}$ of current intensity, for cells with ethanol, and sometimes maximums values of current intensity around 77, 50 and 35 μA . For cells with glycerol were produced $6 \pm 7 \mu\text{A}$ of current intensity, in terms of average values, and sometimes maximums values of current intensity around 23 and 21 μA .

Finally, power density by surface area were determined to assays performed by MET mechanism. The maximal power density produced by surface area was 4.6×10^{-7} and $1.3 \times 10^{-7} \text{ W/cm}^2$, for cells with ethanol and glycerol, respectively. These values can be improved by increase of surface area of bioanode or change some materials that promote the electrons transference.

Lastly, the results of power production are too far from the expected, however it is challenge of Biofuel cells, reach power production in same range than conventional fuel cells.

CHAPTER 6: REFERENCES

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CHAPTER 7: ANNEXES

ANNEX A: CALIBRATION CURVE OF ENZYMATIC ACTIVITY DETERMINATION

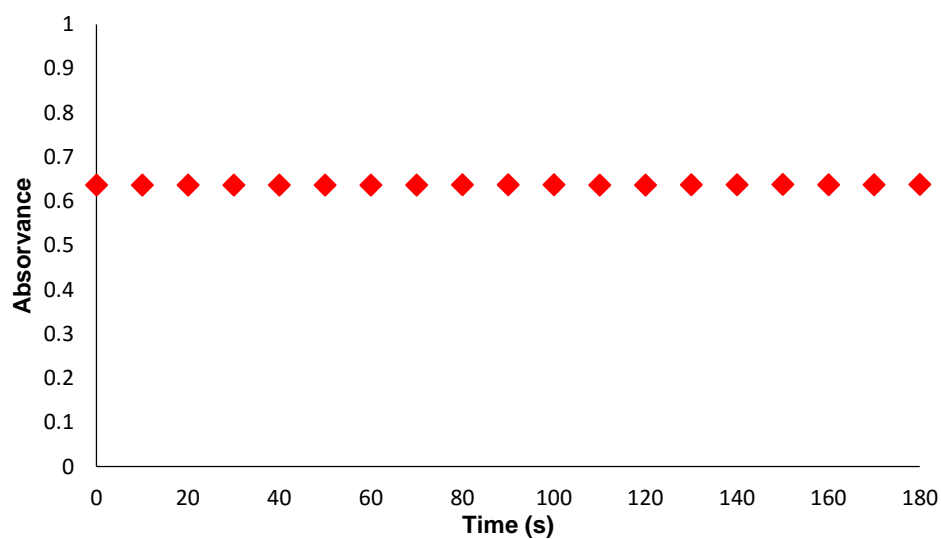


Figure 51: Assay of enzymatic activity performed at controlled temperature 37 °C and in 3 mL cuvette with 70 rpm of stirring. The assays were conducted by, potassium phosphate buffer (37.5 mM, pH 7,3), PMS (60 μ M), DCPIP (35 μ M) and substrate (0.02 M). The absorbance was read at 600 nm during 3 minutes but in absence of enzymes.

ANNEX B: ENZYMATIC ACTIVITY WITH GLUTARALDEHYDE AS SUBSTRATE

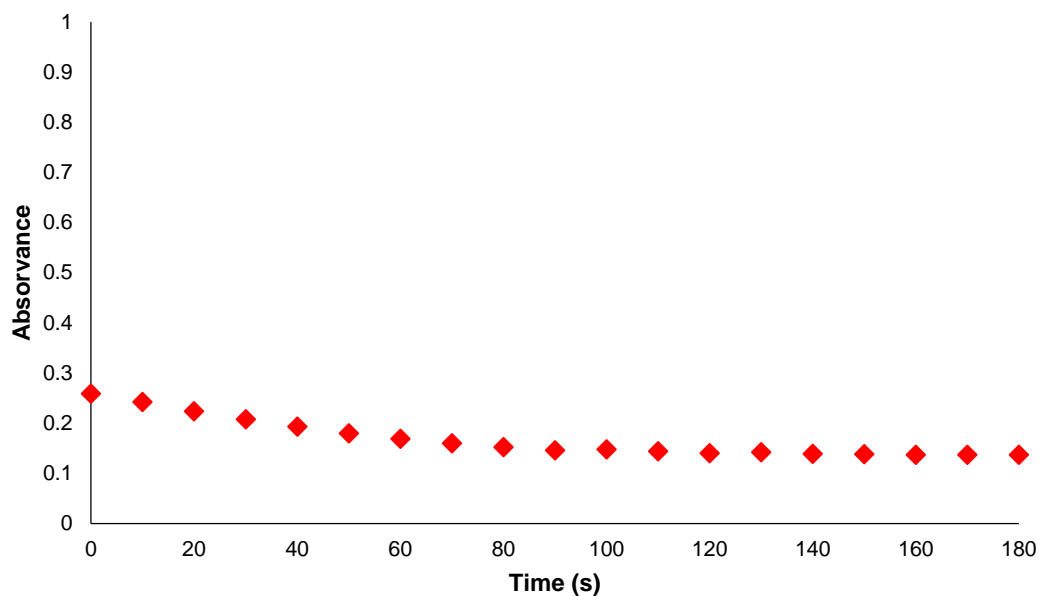


Figure 52: Assay of enzymatic activity performed at controlled temperature 37 °C and in 3 mL cuvette with 70 rpm of stirring. The assays were conducted by, potassium phosphate buffer (37.5 mM, pH 7,3), PMS (60 μ M), DCPIP (35 μ M) and glutaraldehyde (0.2 M). The absorbance was read at 600 nm during 3 minutes but in presence of both PQQ-enzymes.

ANNEX C: EFFECT OF PMS STORAGE ON ENZYMATIC ACTIVITY DETERMINATION

In order to understand stability of PMS, it was performed assays of determination of enzymatic activity in cells, from YPM medium and BM. In Figures 53 and 54 are presented PQQ-ALDH enzyme activity values. The conditions of PMS used are: PMS stored at 4 °C; PMS stored at – 20 °C and a fresh solution of PMS. It is possible to conclude that effect of storage isn't relevant.

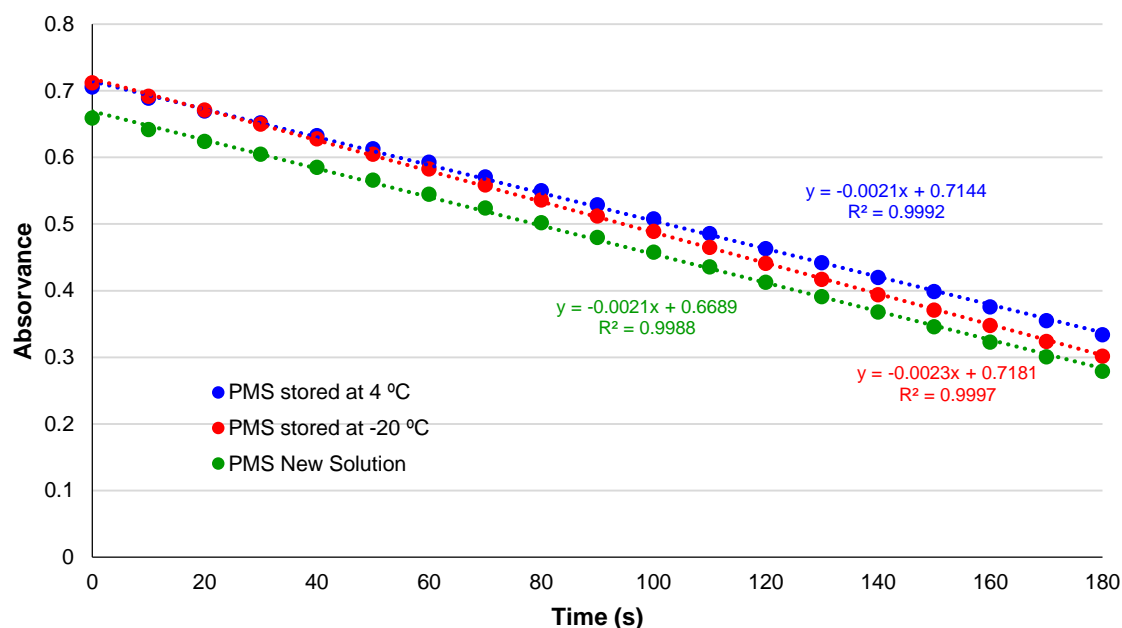


Figure 53: Determination of enzymatic activity of PQQ-ALDH from cells of YPM medium.

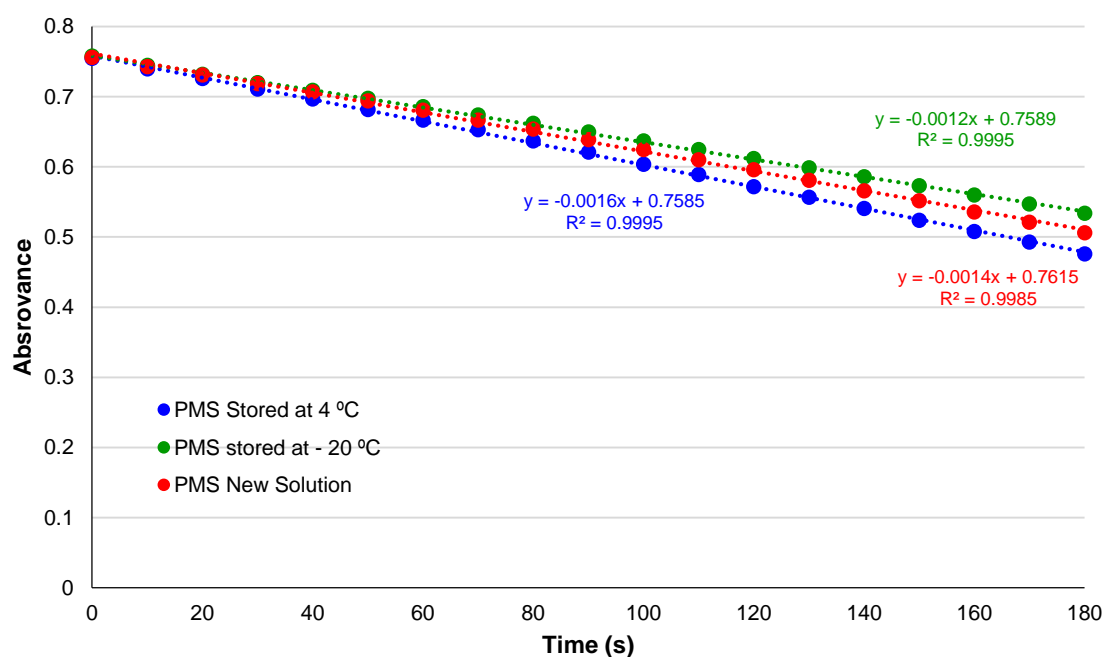


Figure 54: Determination of enzymatic activity of PQQ-ALDH from cells of BM.

ANNEX D: CONTROL VOLTAMMOGRAMS OF DET AND MET

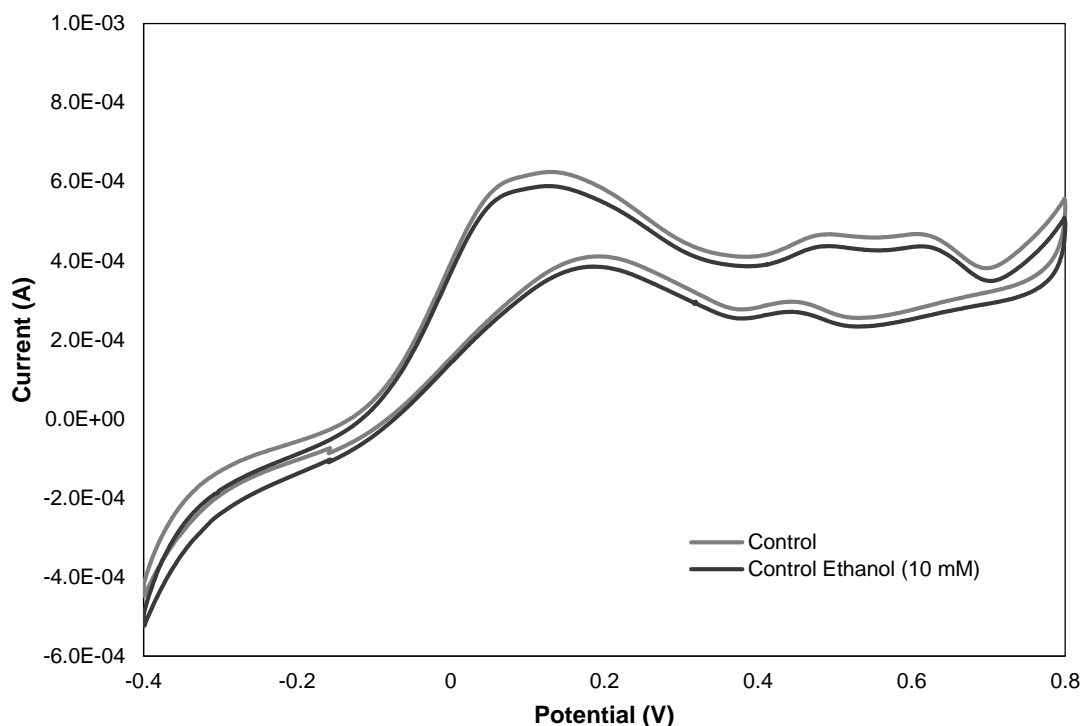


Figure 55: Representative cyclic voltammograms of control of working electrode, i.e., cells free, with 0.5% of Glutaraldehyde on a carbon felt surface. The assays were made based of DET mechanism, in Tris-HCl buffer and CaCl_2 , with 5 / 10 mM ethanol, pH 7.5 at voltage rate of $0.010 \text{ V} \cdot \text{s}^{-1}$

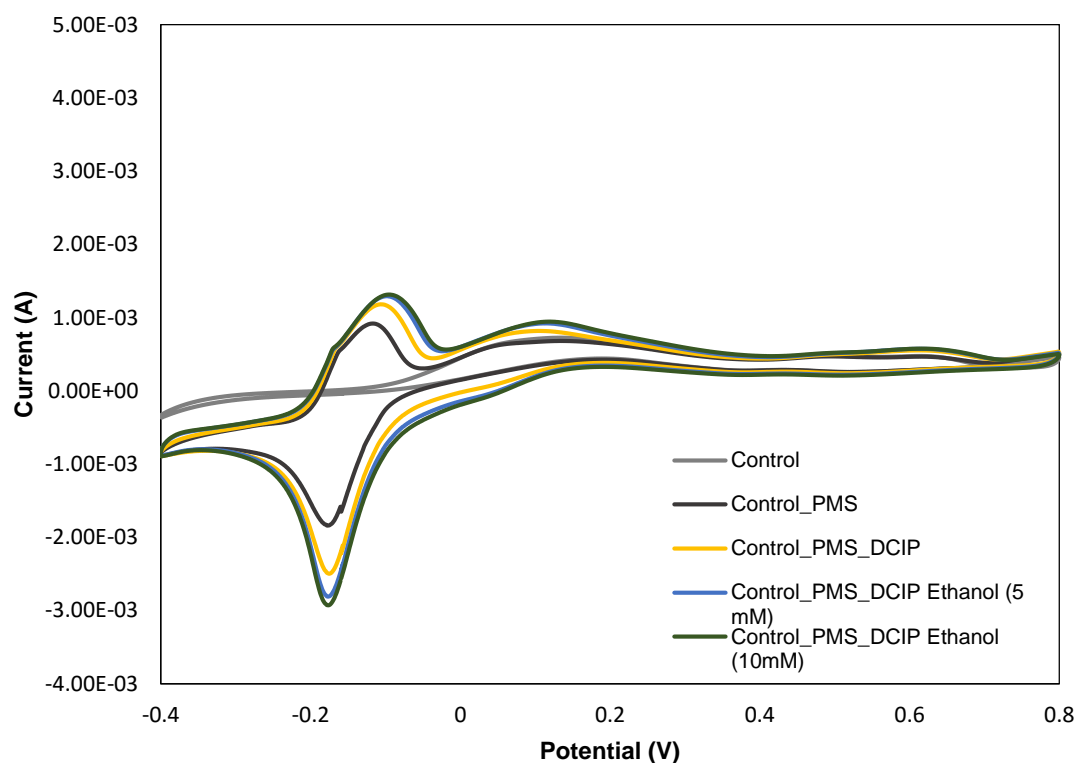


Figure 56: Representative cyclic voltammograms of control of working electrode, i.e., cells free, with 0.5% of Glutaraldehyde on a carbon felt surface. The assays were made based of MET mechanism, in Tris-HCl buffer and CaCl_2 , 35 μM DCPIP and 60 μM PMS, with 5 / 10 mM of glycerol, pH 7.5 at voltage rate of $0.010 \text{ V} \cdot \text{s}^{-1}$.